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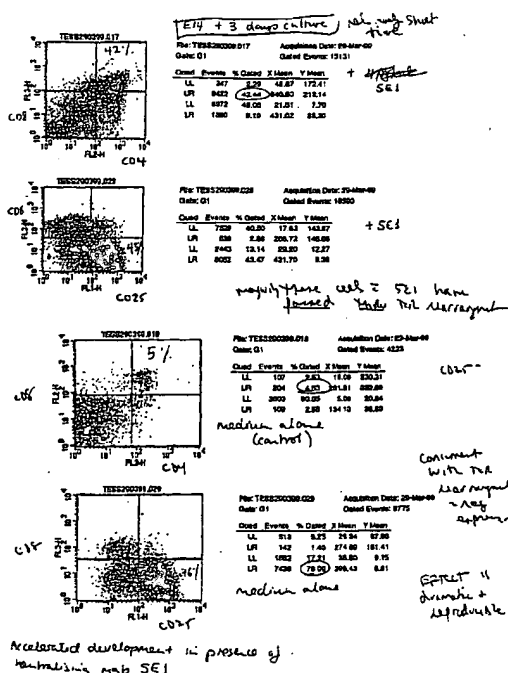
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(54) Title: METHODS AND COMPOSITIONS FOR REGULATING LYMPHOCYTE ACTIVITY



(57) Abstract: The present application is directed to the discovery that hedgehog gene products, and signal transduction pathways involving hedgehog, are involved in maturation of T lymphocytes. Certain aspects of the invention are directed to preparations of hedgehog polypeptides, agonists, antagonists, or other molecules which regulate *patched* or *smoothed* signalling, and their uses as immunomodulatory agents.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Methods and Compositions for Regulating Lymphocyte Activity

Priority Information

This application claims priority to U.S. Provisional Application 60/168,112, filed
5 November 30, 1999.

Background of the Invention

The lymphoid system performs critical functions in animals that include preventing
and combating infection, and surveillance and immune elimination of tumor cells. Loss of
immune function leads to an immunocompromised status that can predispose the host to
10 serious and life-threatening disease. Functional abnormalities may be present in any of
the elements that participate in mediating an immune response, e.g., cellular or humoral
elements such as granulocytes, lymphocytes, complement, antibody, or cytokines.

The immune system is a network of cells adapted to protect the organism against
pathogens and cells that are not recognized as "self." Once the immune system is
15 activated, it enlists the participation of a variety of cells and molecules to mount an
effector function designed to eliminate the "non-self" entity within the body.
Lymphocytes are cells of the immune system that are capable of specifically recognizing
and selectively eliminating foreign entities. By contrast to other cells of the immune
system, such as neutrophils which are considered non-specific in their reactions to
20 invaders, the characteristics of lymphocytes confer specificity, diversity, memory and
self/nonself recognition to the immune response.

There are two major populations of lymphocytes: B lymphocytes and T
lymphocytes. B lymphocytes originate and mature within the bone marrow and are
responsible for formation of antibody molecules. T lymphocytes also arise from the bone
25 marrow but mature in the thymus. There are two major subpopulations of T-cells: T
helper cells and T cytotoxic cells. The two types of T cells can be distinguished by the
presence of one of two membrane glycoproteins, either CD4 or CD8. The T-helper cells
(which express CD4) when activated by antigen-complexes (foreign molecules coupled to
special proteins) respond by secreting various growth factors known collectively as
30 cytokines. These cytokines are signals that activate other cells of the immune system,
including the T-cytotoxic cells. The T-cytotoxic cells (which express CD8) when
activated, proliferate and differentiate into cytotoxic T lymphocytes (CTL) which are able
to monitor for and eliminate from the body pathogenic cells, foreign cells, virus-infected
cells, and tumor cells.

The normal development, maturation and differentiation of T lymphocytes are regulated by various peptide factors

Summary of the Invention

5 The present invention provides methods and compositions for modulating the immune function of an animal by administering an agent which mimics, or alternatively inhibits, *hedgehog*-mediated signal transduction. Thus, the subject method can be used as an immunosuppressive, e.g., by administering a *hedgehog* agonist, or as an immunostimulant, e.g., by administering a *hedgehog* antagonist

10 Wherein the subject method is carried out using a *hedgehog* therapeutic, the *hedgehog* therapeutic preferably a polypeptide including a *hedgehog* portion comprising at least a bioactive extracellular portion of a *hedgehog* protein, e.g., the *hedgehog* portion includes at least 50, 100 or 150 (contiguous) amino acid residues of an N-terminal half of a *hedgehog* protein. In preferred embodiments, the *hedgehog* portion includes at least a
15 portion of the *hedgehog* protein corresponding to a 19kd fragment of the extracellular domain of a *hedgehog* protein.

 In preferred embodiments, the *hedgehog* portion has an amino acid sequence at least 60, 75, 85, or 95 percent identical with a *hedgehog* protein of any of SEQ ID Nos. 10-18 or 20, though sequences identical to those sequence listing entries are also contemplated as
20 useful in the present method. The *hedgehog* portion can be encoded by a nucleic acid which hybridizes under stringent conditions to a nucleic acid sequence of any of SEQ ID Nos. 1-9 or 19, e.g., the *hedgehog* portion can be encoded by a vertebrate *hedgehog* gene, especially a human *hedgehog* gene.

 In other embodiments, the subject method can be carried out by administering a
25 gene activation construct, wherein the gene activation construct is designed to recombine with a genomic *hedgehog* gene of the patient to provide a heterologous transcriptional regulatory sequence operatively linked to a coding sequence of the *hedgehog* gene.

 In still other embodiments, the subject method can be practiced with the administration of a gene therapy construct encoding a *hedgehog* polypeptide. For instance,
30 the gene therapy construct can be provided in a composition selected from a group consisting of a recombinant viral particle, a liposome, and a poly-cationic nucleic acid binding agent,

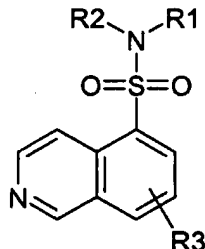
 In yet other embodiments, the subject method can be carried out using a *ptc* therapeutic. An exemplary *ptc* therapeutic is a small organic molecule which binds to a
35 *patched* protein and derepresses *patched*-mediated inhibition of mitosis, e.g., a molecule which binds to *patched* and mimics *hedgehog*-mediated *patched* signal transduction, which

binds to *patched* and regulates *patched*-dependent gene expression. For instance, the binding of the *ptc* therapeutic to *patched* may result in upregulation of *patched* and/or *gli* expression.

In a more generic sense, the *ptc* therapeutic can be a small organic molecule which interacts with MK cells to induce *hedgehog*-mediated *patched* signal transduction, such as by altering the localization, protein-protein binding and/or enzymatic activity of an intracellular protein involved in a *patched* signal pathway. For instance, the *ptc* therapeutic may alter the level of expression of a *hedgehog* protein, a *patched* protein or a protein involved in the intracellular signal transduction pathway of *patched*.

In certain embodiments, the *ptc* therapeutic is an antisense construct which inhibits the expression of a protein which is involved in the signal transduction pathway of *patched* and the expression of which antagonizes *hedgehog*-mediated signals. The antisense construct is preferably an oligonucleotide of about 20-30 nucleotides in length and having a GC content of at least 50 percent.

In other embodiments, the *ptc* therapeutic is an inhibitor of protein kinase A (PKA), such as a 5-isoquinolinesulfonamide. The PKA inhibitor can be a cyclic AMP analog. Exemplary PKA inhibitors include N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, KT5720, 8-bromo-cAMP, dibutyryl-cAMP and PKA Heat Stable Inhibitor isoform α . Another exemplary PKA inhibitor is represented in the general formula:



wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

Brief Description of the Figures

Figures 1-4 illustrate the profile for staining with various markers for T-cell maturity.

Detailed Description of the Invention

I. Overview

The present application is directed to the discovery that hedgehog gene products are able to regulate maturation of T lymphocytes. Certain aspects of the invention are directed to preparations of hedgehog polypeptides, or other molecules which regulate *patched* or *smoothened* signalling, and their uses as immunomodulatory agents against both acquired and hereditary immunological disorders.

For instance, such compositions can be used to increase the population of T-helper cells to optimum levels in the host, e.g. to stimulate the immune system of the animal. Such uses of the subject compositions can be used in the treatment of bacterial or viral infections, as well as to help the body fight against cancer cells. Alternatively, these substances also enable the host to adjust to diseases arising from disarrangement of self-recognition processes in which there is excessive attack by host T-cells against endogenous tissues. In such instances, the subject compositions can be used to reduce T-cell population so that the signs and symptoms of self-directed inflammatory (autoimmune) diseases such rheumatoid arthritis and multiple sclerosis are ameliorated.

As described herein, *hedgehog* proteins inhibit maturation of T lymphocytes. Based upon its inhibitory effect, the administration of hedgehog or *ptc* therapeutics (defined *infra*) is suggested herein as a treatment for several types of immunological disorders involving

unwanted activation of cellular immunity, e.g., graft rejection, autoimmune disorders, and the like. In other embodiments, inhibitors of hedgehog signalling pathways can be used as immunostimulatory agents, e.g., to counteract the effects of any endogenous activation of a hedgehog/ptc pathway.

5 In general, the method of the present invention comprises administering to animal, or to cultured lymphocytes *in vitro*, an amount of a hedgehog or ptc therapeutic which produces a non-toxic response by the cell of inhibition of maturation (in the case of a hedgehog agonist) or promotion of T cell maturation (in the case of a hedgehog antagonist). The subject method can be carried out on cells which may be either dispersed in culture or a
10 part of an intact tissue or organ. Moreover, the method can be performed on cells which are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*).

In one aspect, the present invention provides pharmaceutical preparations and methods for treating or preventing hyperimmune or hypoimmune disorders utilizing, as an active ingredient, a *hedgehog* polypeptide or a mimetic thereof. The invention also relates
15 to methods of controlling the functional performance of T cells by use of the pharmaceutical preparations of the invention.

The subject *hedgehog* treatments are effective on both human and animal subjects afflicted with these conditions. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes.
20 Examples are dogs, cats, cattle, horses, sheep, hogs and goats.

Without wishing to be bound by any particular theory, the inhibitory effect of *hedgehog* on T cell maturation may be due at least in part to the ability of hedgehog proteins to antagonize (directly or indirectly) *patched*-mediated regulation of gene expression and other physiological effects mediated by that protein. The *patched* gene
25 product, a cell surface protein, is understood to signal through a pathway which causes transcriptional repression of members of the Wnt and Dpp/BMP families of morphogens, proteins which impart positional information. In other tissue, the introduction of *hedgehog* relieves (derepresses) this inhibition conferred by *patched*, allowing expression of particular gene programs.

30 Recently, it has been reported that mutations in the human version of *patched*, a gene first identified in a fruit fly developmental pathway, cause a hereditary skin cancer and may contribute to sporadic skin cancers. See, for example, Hahn et al. (1996) *Cell* 86:841-851; and Johnson et al. (1996) *Science* 272:1668-1671. The demonstration that nevoid basal-cell carcinoma (NBCC) results from mutations in the human *patched* gene provided
35 an example of the roles *patched* plays in post-embryonic development. These observations have led the art to understand one activity of *patched* to be a tumor suppressor gene, which may act by inhibiting proliferative signals from *hedgehog*. Our observations set forth below

reveal potential new roles for the *hedgehog/patched* pathway in maintenance of mature T-cells and other lymphocytic cell lines. Accordingly, the present invention contemplates the use of other agents which are capable of mimicking or antagonizing, depending on the desired consequence, the effect of the *hedgehog* protein on *patched* signalling, e.g., as may
5 be identified from the drug screening assays described below.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

10 The term "hedgehog therapeutic" refers to various forms of hedgehog polypeptides, as well as peptidomimetics, which can modulate the proliferation/differentiation state of lymphocytes, by, as will be clear from the context of individual examples, mimicking or potentiating (agonizing) or inhibiting (antagonizing) the effects of a naturally-occurring *hedgehog* protein. A *hedgehog* therapeutic which mimics or potentiates the activity of a
15 wild-type hedgehog protein is a "hedgehog agonist". Conversely, a *hedgehog* therapeutic which inhibits the activity of a wild-type hedgehog protein is a "hedgehog antagonist".

In particular, the term "hedgehog polypeptide" encompasses preparations of *hedgehog* proteins and peptidyl fragments thereof, both agonist and antagonist forms as the specific context will make clear.

20 As used herein the term "bioactive fragment of a *hedgehog* protein" refers to a fragment of a full-length *hedgehog* polypeptide, wherein the fragment specifically agonizes or antagonizes inductive events mediated by wild-type *hedgehog* proteins. The *hedgehog* bioactive fragment preferably is a soluble extracellular portion of a *hedgehog* protein, where solubility is with reference to physiologically compatible solutions. Exemplary bioactive
25 fragments are described in PCT publications WO 95/18856 and WO 96/17924.

The term "ptc therapeutic" refers to agents which either (i) mimic the effect of *hedgehog* proteins on *patched* signalling, e.g., which antagonize the cell-cycle inhibitory and/or T-cell maturation promoting activity of *patched*, or (ii) activate or potentiate *patched* signalling. In other embodiments, the ptc therapeutic can be a *hedgehog* antagonist. The
30 ptc therapeutic can be, e.g., a peptide, a nucleic acid, a carbohydrate, a small organic molecule, or natural product extract (or fraction thereof).

An "effective amount" of, e.g., a hedgehog or ptc therapeutic, with respect to the subject method of treatment, refers to an amount of a composition, e.g., a hedgehog polypeptide, in a preparation which, when applied as part of a desired dosage regimen
35 brings enhances or inhibits (as the case may be) T-cell maturation, relative to the absence of

the hedgehog or ptc therapeutic, according to clinically acceptable standards for the disorder to be treated.

A "patient" or "subject" to be treated by the subject method can mean either a human or non-human animal.

- 5 The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

"Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous at that position. A percentage of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with an hedgehog sequence of the present invention.

- 20 The term "corresponds to", when referring to a particular polypeptide or nucleic acid sequence is meant to indicate that the sequence of interest is identical or homologous to the reference sequence to which it is said to correspond.

The terms "recombinant protein", "heterologous protein" and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is inserted into a suitable expression construct which is in turn used to transform a host cell to produce the heterologous protein. That is, the polypeptide is expressed from a heterologous nucleic acid.

- 30 A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding a *hedgehog* polypeptide with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of *hh* protein. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula $(X)_n-(hh)_m-(Y)_n$, wherein *hh* represents all or a portion of the *hedgehog* protein, X and Y each independently represent an amino acid sequences which are not naturally found as a polypeptide chain contiguous with the *hedgehog* sequence, m is an integer greater than or equal to 1, and each occurrence
- 35

of n is, independently, 0 or an integer greater than or equal to 1 (n and m are preferably no greater than 5 or 10).

The term "CD4⁺ lymphocyte" is intended to mean a lymphocyte having a plurality of cell surface CD4 molecules as evidenced by binding of an antibody specific for CD4 to the cell surface, e.g., monoclonal antibody OKT4 (Ortho Diagnostics, Piscataway, N.J.).

The term "CD8⁺ lymphocyte" is intended to mean a lymphocyte having a plurality of cell surface CD8 molecules as evidenced by binding of an antibody specific for CD8 to the cell surface, e.g., monoclonal antibody OKT8 (Ortho Diagnostics, Piscataway, N.J.).

"Peripheral blood leukocytes", abbreviated PBL, are intended to mean the cellular components of the immune system in blood, e.g., lymphocytes, monocytes, eosinophils, basophils, neutrophils, plasma cells, mast cell precursors, and the like.

"Lymphocytes" are intended to encompass T-lymphocytes, (also referred to as T-cells), B-lymphocytes (also referred to as B-cells), natural killer-lymphocytes (NK-cells), cytotoxic T lymphocytes (CTL), T-helper lymphocytes, delta gamma -T-cell receptor bearing cells as well as precursors and activated derivatives thereof

"Activated lymphocyte" is intended to mean that subset of lymphocytes which has been i) exposed to a stimulus, and ii) has been triggered to change from a metabolically-quiescent cell into a cell with increased protein synthesis and/or DNA and RNA synthesis and possibly cell division. Illustrative stimuli and triggering pathways leading to activated lymphocytes include interaction of T cell receptors with antigens, interaction of interleukin receptors with interleukins, interaction of growth factor receptors with growth factors, interaction of lymphocyte cell surface determinants with antibody (e.g., anti-CD2) or mitogens (e.g., PHA), and the like.

"Subject in need thereof" is intended to mean a mammal having one or more clinical or laboratory indicia of a disorder or disease. The subject may exhibit clinical disease activity or may have a subclinical or latent infection. Subjects in need thereof include human and non-human primates, mammals, domestic animals, livestock, and the like, e.g., dogs, cats, rodents, birds, horses, cows, pigs and fish.

III. Exemplary Applications of Method and Compositions

According to the invention, preparations with ptc and hedgehog therapeutics are used in immunoregulatory disorders and diseases in animals and humans, for the prevention or prophylaxis, control, diagnosis or treatment thereof.

The subject method has wide applicability to the treatment or prophylaxis of disorders affecting the regulation of lymphocytes, particularly maturation and/or activation

of T lymphocytes. In general, the method can be characterized as including a step of administering to an animal an amount of a *ptc* or hedgehog therapeutic effective to alter the proliferative and/or differentiation state of treated lymphocytes. Such therapeutic compositions may be useful in treatments designed to modulate, e.g., increase or decrease, an immunological response. Such diseases and conditions include, but are not limited to, infection (such as bacterial or viral infection), metabolic disease such as diabetes, nutritional deficiency, toxic agents, graft rejection or other hyperacute response, or autoimmune disorders. The goals of treatment in each case can be twofold: (1) to eliminate the cause of the disease or unwanted immunological response, and (2) to relieve its symptoms.

In view of their immunosuppressant activity, the hedgehog proteins (and agonists thereof) are suitable for preventing and treating diseases and conditions which require a temporary or permanent reduction or suppression of an immune response. In particular, their use extends to suppressing the activation of the proliferation of lymphocytes or cytotoxic T-cells and/or immunocytes, e.g. for preventing or treating autoimmune diseases such as diseases of the rheumatic type, multiple sclerosis, psoriasis, atopic dermatitis, or for preventing the rejection of transplanted tissues or organs such as kidneys, heart, lungs, bone marrow, spleen, skin or cornea, in undesirable reactions during or after transfusions, allergic diseases, particularly those which affect the gastrointestinal tract and which may take the form of an inflammation, or inflammatory, proliferative and hyperproliferative diseases and cutaneous manifestations of immunological disorders such as eczematous dermatitis, urticaria, vasculitis and scleroderma.

Thus, it is particularly advantageous to use immunosuppressive forms of the subject hedgehog and *ptc* therapeutics clinically for the disorders, diseases and conditions described above, i.e. when it is desirable to achieve immunosuppression in an animal or human body.

Depending on the nature and cause of the disease or disorder to be treated or the condition which is to be influenced in an animal or human body, it may be desirable to administer the hedgehog or *ptc* therapeutic preparation systemically, locally or topically to the tissue or organ in question. Systemic action is desirable, for example, when various organs or organ systems are in need of treatment, as is the case for example in systemic autoimmune diseases or allergies or in transplants of large, foreign organs or tissues. By contrast, a local effect would be considered if only local manifestations of an immunological occurrence had to be treated, e.g. in small transplants of skin or cornea or in cases of local dermatitis.

Depending on the duration and intensity of the immunosuppressant activity required, the hedgehog or *ptc* therapeutic preparations may be given one or more times a

day, as well as intermittently, over a period of several days, weeks or months and in various dosages.

In still other embodiments, antagonist (lymphocyte maturation promoting) forms of the subject ptc and hedgehog therapeutics can be used to treat disorders involving hyp immunity, e.g., immunosuppressed or immunocompromised patients. For instance, the effect of hedgehog-induced immunosuppression, e.g., resulting from endogenous or heterologous activation of the hedgehog signalling pathway, can be counteracted using antagonist forms of the subject ptc and hedgehog therapeutics.

Thus, the subject method contemplates the treatment of immunocompromised subjects to increase one or more indicia of cell mediated immunity (CMI), humoral immunity, or innate resistance to infection, by administering pharmaceutical preparations of an activating ptc or hedgehog therapeutic. In certain embodiments, such immunity-promoting activities of antagonists forms of the subject ptc and hedgehog therapeutics can be identified, e.g., by i) increased E-rosette forming cells (E-RFC) in thymocyte cultures after incubation with the subject ptc or hedgehog therapeutic agents; ii) increased E-RFC in cultures of thymocytes from aged animals after incubation with the subject ptc or hedgehog therapeutic agents; and, iii) increased expression of OKT 4< + > in cultures of human peripheral blood T-lymphocytes from patients with secondary immunodeficiency syndromes following treatment with the subject ptc or hedgehog therapeutic agents. Increased expression of CD2 and CD4 accessory molecules on T-lymphocytes is compatible with a heighten the state of innate or induced immunity to infection, e.g., by upregulating T-helper and cytotoxic T-lymphocytes to respond to lower levels of antigen.

Immunodeficiency states fall into three general etiologic categories. First, there is immunosuppression that occurs as a consequence of disease processes. Second, there are immunodeficiencies that arise because of therapy for other diseases, so-called iatrogenic immunodeficiencies. Third, immunodeficiencies may result from direct attack of T-lymphocytes by the human immunodeficiency virus (HIV) that causes the acquired immunodeficiency syndrome (AIDS).

Common disease processes that lead to immunodeficiency are malnutrition, neoplasias, aging, and infections. Malnourished people, patients with advanced widespread cancers and people with debilitating illnesses become sick and die more often because impaired cell-mediated and humoral immune responses increase susceptibility to infections by a variety of organisms. A state of generalized deficiency in immune responses is called anergy. Various types of infections, especially viral infections, lead to immunosuppression. A drug, such as a maturation-promoting form of a Ptc or hedgehog therapeutic, e.g., capable of making the T-helper lymphocyte components of the immune system more robust, will be

an important therapeutic agent for increasing the resistance of the patient to infections. For example, Ptc or hedgehog therapeutic or its analogs, may be:

administered to patients, especially older patients, before or just after admissions to hospitals in order to reduce the risks of nosocomial (hospital-induced) infections, a
5 common and severe clinical problem

administered to burn victims, because such individuals are especially prone to infections

administered to patients in anticipation of epidemic infections, for example, in conjunction with influenza vaccinations or hepatitis vaccinations, to invigorate the immune
10 response to pathogens

administered to patients with asymptomatic viral infections, in order to enhance immune surveillance of pathogenic organisms and reduce the likelihood of recurrence of disease, for example, for individuals who are carriers of herpes viruses, varicella viruses, hepatitis viruses and HIV.

15 Iatrogenic immunosuppression is most often due to drug therapies which either kill or functionally inactivate lymphocytes. Various chemotherapeutic drugs are administered to cancer patients, and these drugs are usually cytotoxic to both mature and developing lymphocytes as well as to granulocyte and monocyte precursors. Thus, cancer chemotherapy is almost always accompanied by a period of immunosuppression and
20 increased risk of infections. Radiation treatment of cancer carries the same risks. Medications (granulocyte-colony stimulating factor) exist for increasing neutrophils in blood to combat infections that occur after cancer chemotherapy, but no medications are currently used for restoring lymphocytic functions. Major surgery, for example repair of aneurysms or by-pass operations, also decrease immune function in humans. The reasons
25 for the decline in blood lymphocytes that occur because of major surgery are not clear, but an agent that elevates lymphocyte functions in such patients have therapeutic value in decreasing the likelihood of infections.

One final form of acquired immunosuppression that should be mentioned results from the absence of a spleen, caused by surgical removal of the organ after trauma or for
30 the treatment of certain hematologic diseases or as a result of infarction in sickle cell disease. Patients without spleens are more susceptible to infections by some organisms, particularly encapsulated bacteria such as *Streptococcus pneumoniae*. The spleen is apparently required for the induction of protective humoral immune responses to such organisms. The subject Ptc or hedgehog therapeutics can help individuals without a spleen
35 or without a thymus in resistance against infection by micro-organisms.

IV. Exemplary hedgehog therapeutic compounds.

The *hedgehog* therapeutic compositions of the subject method can be generated by any of a variety of techniques, including purification of naturally occurring proteins, recombinantly produced proteins and synthetic chemistry. Polypeptide forms of the

5 hedgehog therapeutics are preferably derived from vertebrate hedgehog proteins, e.g., have sequences corresponding to naturally occurring hedgehog proteins, or fragments thereof, from vertebrate organisms. However, it will be appreciated that the hedgehog polypeptide can correspond to a hedgehog protein (or fragment thereof) which occurs in any metazoan organism.

10 The various naturally-occurring *hedgehog* proteins from which the subject therapeutics can be derived are characterized by a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. *et al.* (1992) *Cell* 71:33-50; Tabata, T. *et al.* (1992) *Genes Dev.* 2635-2645; Chang, D.E. *et al.* (1994) *Development* 120:3339-3353),

15 *hedgehog* precursor proteins naturally undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) *Science* 266:1528-1537; Porter *et al.* (1995) *Nature* 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) *supra*; Tabata *et al.* (1992) *supra*; Chang *et al.* (1994) *supra*; Lee *et al.* (1994) *supra*; Bumcrot,

20 D.A., *et al.* (1995) *Mol. Cell. Biol.* 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) *Curr. Biol.* 5:944-955; Lai, C.J. *et al.* (1995) *Development* 121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra*; Mart', E. *et al.* (1995) *Development*

25 121:2537-2547; Roelink, H. *et al.* (1995) *Cell* 81:445-455). Cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of *hedgehog* encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) *Cell* 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the *hedgehog*

30 precursor protein proceeds through an internal thioester intermediate which subsequently is cleaved in a nucleophilic substitution. It is suggested that the nucleophile is a small lipophilic molecule, more particularly cholesterol, which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), tethering it to the cell surface.

The vertebrate family of *hedgehog* genes includes at least four members, e.g.,

35 paralogs of the single drosophila *hedgehog* gene (SEQ ID No. 19). Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle *hedgehog* (*Thh*), appears specific to fish.

According to the appended sequence listing, (see also Table 1) a chicken *Shh* polypeptide is encoded by SEQ ID No:1; a mouse *Dhh* polypeptide is encoded by SEQ ID No:2; a mouse *Ihh* polypeptide is encoded by SEQ ID No:3; a mouse *Shh* polypeptide is encoded by SEQ ID No:4 a zebrafish *Shh* polypeptide is encoded by SEQ ID No:5; a human *Shh* polypeptide is encoded by SEQ ID No:6; a human *Ihh* polypeptide is encoded by SEQ ID No:7; a human *Dhh* polypeptide is encoded by SEQ ID No. 8; and a zebrafish *Thh* is encoded by SEQ ID No. 9.

Table 1
Guide to *hedgehog* sequences in Sequence Listing

	Nucleotide	Amino Acid
Chicken <i>Shh</i>	SEQ ID No. 1	SEQ ID No. 10
Mouse <i>Dhh</i>	SEQ ID No. 2	SEQ ID No. 11
Mouse <i>Ihh</i>	SEQ ID No. 3	SEQ ID No. 12
Mouse <i>Shh</i>	SEQ ID No. 4	SEQ ID No. 13
Zebrafish <i>Shh</i>	SEQ ID No. 5	SEQ ID No. 14
Human <i>Shh</i>	SEQ ID No. 6	SEQ ID No. 15
Human <i>Ihh</i>	SEQ ID No. 7	SEQ ID No. 16
Human <i>Dhh</i>	SEQ ID No. 8	SEQ ID No. 17
Zebrafish <i>Thh</i>	SEQ ID No. 9	SEQ ID No. 18
Drosophila <i>HH</i>	SEQ ID No. 19	SEQ ID No. 20

In addition to the sequence variation between the various *hedgehog* homologs, the *hedgehog* proteins are apparently present naturally in a number of different forms, including a pro-form, a full-length mature form, and several processed fragments thereof. The pro-form includes an N-terminal signal peptide for directed secretion of the extracellular domain, while the full-length mature form lacks this signal sequence.

As described above, further processing of the mature form occurs in some instances to yield biologically active fragments of the protein. For instance, *sonic hedgehog* undergoes additional proteolytic processing to yield two peptides of approximately 19 kDa and 27 kDa, the 19kDa fragment corresponding to an proteolytic N-terminal portion of the mature protein.

In addition to proteolytic fragmentation, the vertebrate *hedgehog* proteins can also be modified post-translationally, such as by glycosylation and/or addition of lipophilic moieties, such as stents, fatty acids, etc., though bacterially produced (e.g. unmodified) forms of the proteins still maintain certain of the bioactivities of the native protein. Bioactive fragments of *hedgehog* polypeptides of the present invention have been generated and are described in great detail in, e.g., PCT publications WO 95/18856 and WO 96/17924.

There are a wide range of lipophilic moieties with which hedgehog polypeptides can be derivatived. The term "lipophilic group", in the context of being attached to a hedgehog polypeptide, refers to a group having high hydrocarbon content thereby giving the group high affinity to lipid phases. A lipophilic group can be, for example, a relatively long chain alkyl or cycloalkyl (preferably n-alkyl) group having approximately 7 to 30 carbons. The alkyl group may terminate with a hydroxy or primary amine "tail". To further illustrate, lipophilic molecules include naturally-occurring and synthetic aromatic and non-aromatic moieties such as fatty acids, sterols, esters and alcohols, other lipid molecules, cage structures such as adamantane and buckminsterfullerenes, and aromatic hydrocarbons such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.

In one embodiment, the hedgehog polypeptide is modified with one or more sterol moieties, such as cholesterol. See, for example, PCT publication WO 96/17924. In certain embodiments, the cholesterol is preferably added to the C-terminal glycine where the hedgehog polypeptide corresponds to the naturally-occurring N-terminal proteolytic fragment.

In another embodiment, the hedgehog polypeptide can be modified with a fatty acid moiety, such as a myristoyl, palmitoyl, stearoyl, or arachidoyl moiety. See, e.g., Pepinsky et al. (1998) J Biol. Chem 273: 14037.

In addition to those effects seen by cholesterol-addition to the C-terminus or fatty acid addition to the N-terminus of extracellular fragments of the protein, at least certain of the biological activities of the hedgehog gene products are unexpectedly potentiated by derivatization of the protein with lipophilic moieties at other sites on the protein and/or by moieties other than cholesterol or fatty acids. Certain aspects of the invention are directed to the use of preparations of hedgehog polypeptides which are modified at sites other than N-terminal or C-terminal residues of the natural processed form of the protein, and/or which are modified at such terminal residues with lipophilic moieties other than a sterol at the C-terminus or fatty acid at the N-terminus.

Particularly useful as lipophilic molecules are alicyclic hydrocarbons, saturated and unsaturated fatty acids and other lipid and phospholipid moieties, waxes, cholesterol, isoprenoids, terpenes and polycyclic hydrocarbons including adamantane and buckminsterfullerenes, vitamins, polyethylene glycol or oligoethylene glycol, (C1-C18)-alkyl phosphate diesters, -O-CH₂-CH(OH)-O-(C12-C18)-alkyl, and in particular conjugates with pyrene derivatives. The lipophilic moiety can be a lipophilic dye suitable for use in the invention include, but are not limited to, diphenylhexatriene, Nile Red, N-phenyl-1-naphthylamine, Prodan, Laurodan, Pyrene, Perylene, rhodamine, rhodamine B, tetramethylrhodamine, Texas Red, sulforhodamine, 1,1'-didodecyl-

3,3,3',3'tetramethylindocarbocyanine perchlorate, octadecyl rhodamine B and the BODIPY dyes available from Molecular Probes Inc.

Other exemplary lipophilic moieties include aliphatic carbonyl radical groups include 1- or 2-adamantylacetyl, 3-methyladamant-1-ylacetyl, 3-methyl-3-bromo-1-
 5 adamantylacetyl, 1-decalinacetyl, camphoracetyl, camphaneacetyl, noradamantylacetyl, norbornaneacetyl, bicyclo[2.2.2.]-oct-5-eneacetyl, 1-methoxybicyclo[2.2.2.]-oct-5-ene-2-carbonyl, cis-5-norbornene-endo-2,3-dicarbonyl, 5-norbornene-2-ylacetyl, (1R)-(-)-myrtentaneacetyl, 2-norbornaneacetyl, anti-3-oxo-tricyclo[2.2.1.0<2,6>]-heptane-7-carbonyl, decanoyl, dodecanoyl, dodecenoyl, tetradecadienoyl, decynoyl or dodecynoyl.

10 The hedgehog polypeptide can be linked to the hydrophobic moiety in a number of ways including by chemical coupling means, or by genetic engineering.

There are a large number of chemical cross-linking agents that are known to those skilled in the art. For the present invention, the preferred cross-linking agents are heterobifunctional cross-linkers, which can be used to link the hedgehog polypeptide and
 15 hydrophobic moiety in a stepwise manner. Heterobifunctional cross-linkers provide the ability to design more specific coupling methods for conjugating to proteins, thereby reducing the occurrences of unwanted side reactions such as homo-protein polymers. A wide variety of heterobifunctional cross-linkers are known in the art. These include: succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), m-
 20 Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-succinimidylloxycarbonyl-a-methyl-a-(2-pyridyldithio)-toluene (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP). Those
 25 cross-linking agents having N-hydroxysuccinimide moieties can be obtained as the N-hydroxysulfosuccinimide analogs, which generally have greater water solubility. In addition, those cross-linking agents having disulfide bridges within the linking chain can be synthesized instead as the alkyl derivatives so as to reduce the amount of linker cleavage *in vivo*.

30 In addition to the heterobifunctional cross-linkers, there exists a number of other cross-linking agents including homobifunctional and photoreactive cross-linkers. Disuccinimidyl suberate (DSS), bismaleimido-hexane (BMH) and dimethyl-pimelimidate-2 HCl (DMP) are examples of useful homobifunctional cross-linking agents, and bis-[β-(4-azidosalicylamido)ethyl]disulfide (BASED) and N-succinimidyl-6(4'-azido-2'-nitrophenyl-amino)hexanoate (SANPAH) are examples of useful photoreactive cross-linkers for use in
 35 this invention. For a recent review of protein coupling techniques, see Means et al. (1990) *Bioconjugate Chemistry* 1:2-12, incorporated by reference herein.

One particularly useful class of heterobifunctional cross-linkers, included above, contain the primary amine reactive group, N-hydroxysuccinimide (NHS), or its water soluble analog N-hydroxysulfosuccinimide (sulfo-NHS). Primary amines (lysine epsilon groups) at alkaline pH's are unprotonated and react by nucleophilic attack on NHS or sulfo-NHS esters. This reaction results in the formation of an amide bond, and release of NHS or sulfo-NHS as a by-product.

Another reactive group useful as part of a heterobifunctional cross-linker is a thiol reactive group. Common thiol reactive groups include maleimides, halogens, and pyridyl disulfides. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.5-7.5) conditions. Halogens (iodoacetyl functions) react with -SH groups at physiological pH's. Both of these reactive groups result in the formation of stable thioether bonds.

The third component of the heterobifunctional cross-linker is the spacer arm or bridge. The bridge is the structure that connects the two reactive ends. The most apparent attribute of the bridge is its effect on steric hindrance. In some instances, a longer bridge can more easily span the distance necessary to link two complex biomolecules. For instance, SMPB has a span of 14.5 angstroms.

Preparing protein-protein conjugates using heterobifunctional reagents is a two-step process involving the amine reaction and the sulfhydryl reaction. For the first step, the amine reaction, the protein chosen should contain a primary amine. This can be lysine epsilon amines or a primary alpha amine found at the N-terminus of most proteins. The protein should not contain free sulfhydryl groups. In cases where both proteins to be conjugated contain free sulfhydryl groups, one protein can be modified so that all sulfhydryls are blocked using for instance, N-ethylmaleimide (see Partis et al. (1983) J. Pro. Chem. 2:263, incorporated by reference herein). Ellman's Reagent can be used to calculate the quantity of sulfhydryls in a particular protein (see for example Ellman et al. (1958) Arch. Biochem. Biophys. 74:443 and Riddles et al. (1979) Anal. Biochem. 94:75, incorporated by reference herein).

The reaction buffer should be free of extraneous amines and sulfhydryls. The pH of the reaction buffer should be 7.0-7.5. This pH range prevents maleimide groups from reacting with amines, preserving the maleimide group for the second reaction with sulfhydryls.

The NHS-ester containing cross-linkers have limited water solubility. They should be dissolved in a minimal amount of organic solvent (DMF or DMSO) before introducing the cross-linker into the reaction mixture. The cross-linker/solvent forms an emulsion which will allow the reaction to occur.

The sulfo-NHS ester analogs are more water soluble, and can be added directly to the reaction buffer. Buffers of high ionic strength should be avoided, as they have a tendency to "salt out" the sulfo-NHS esters. To avoid loss of reactivity due to hydrolysis, the cross-linker is added to the reaction mixture immediately after dissolving the protein solution.

The reactions can be more efficient in concentrated protein solutions. The more alkaline the pH of the reaction mixture, the faster the rate of reaction. The rate of hydrolysis of the NHS and sulfo-NHS esters will also increase with increasing pH. Higher temperatures will increase the reaction rates for both hydrolysis and acylation.

Once the reaction is completed, the first protein is now activated, with a sulfhydryl reactive moiety. The activated protein may be isolated from the reaction mixture by simple gel filtration or dialysis. To carry out the second step of the cross-linking, the sulfhydryl reaction, the lipophilic group chosen for reaction with maleimides, activated halogens, or pyridyl disulfides must contain a free sulfhydryl. Alternatively, a primary amine may be modified with to add a sulfhydryl

In all cases, the buffer should be degassed to prevent oxidation of sulfhydryl groups. EDTA may be added to chelate any oxidizing metals that may be present in the buffer. Buffers should be free of any sulfhydryl containing compounds.

Maleimides react specifically with -SH groups at slightly acidic to neutral pH ranges (6.5-7.5). A neutral pH is sufficient for reactions involving halogens and pyridyl disulfides. Under these conditions, maleimides generally react with -SH groups within a matter of minutes. Longer reaction times are required for halogens and pyridyl disulfides.

The first sulfhydryl reactive-protein prepared in the amine reaction step is mixed with the sulfhydryl-containing lipophilic group under the appropriate buffer conditions. The conjugates can be isolated from the reaction mixture by methods such as gel filtration or by dialysis.

Exemplary activated lipophilic moieties for conjugation include: N-(1-pyrene)maleimide; 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide; fluorescein-5-maleimide; N-(4-(6-dimethylamino-2-benzofuranyl)phenyl)maleimide; benzophenone-4-maleimide; 4-dimethylaminophenylazophenyl-4'-maleimide (DABMI), tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine Red™ C2 maleimide, N-(5-aminopentyl)maleimide, trifluoroacetic acid salt, N-(2-aminoethyl)maleimide, trifluoroacetic acid salt, Oregon Green™ 488 maleimide, N-(2-(((4-azido-2,3,5,6-tetrafluorobenzoyl)amino)ethyl)dithio)ethyl)maleimide (TFPAM-SS1), 2-(1-(3-dimethylaminopropyl)-indol-3-yl)-3-(indol-3-yl) maleimide (bisindolylmaleimide; GF 109203X), BODIPY® FL N-(2-aminoethyl)maleimide, N-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM), Alexa™ 488 C5 maleimide, Alexa™ 594 C5

maleimide, sodium salt N-(1-pyrene)maleimide, 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide, fluorescein-5-maleimide, N-(4-(6-dimethylamino-2-benzofuranyl)phenyl)maleimide, benzophenone-4-maleimide, 4-dimethylaminophenylazophenyl-4'-maleimide, 1-(2-maleimidylethyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium methanesulfonate, tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine Red™ C2 maleimide, N-(5-aminopentyl)maleimide, N-(2-aminoethyl)maleimide, N-(2-(((4-azido-2,3,5,6-tetrafluoro)benzoyl)amino)ethyl)dithioethylmaleimide, 2-(1-(3-dimethylaminopropyl)-indol-3-yl)-3-(indol-3-yl) maleimide, N-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (DACM), 11H-Benzo[a]fluorene, Benzo[a]pyrene.

In one embodiment, the hedgehog polypeptide can be derivatized using pyrene maleimide, which can be purchased from Molecular Probes (Eugene, Oreg.), e.g., N-(1-pyrene)maleimide or 1-pyrenemethyl iodoacetate (PMIA ester).

For those embodiments wherein the hydrophobic moiety is a polypeptide, the modified hedgehog polypeptide of this invention can be constructed as a fusion protein, containing the hedgehog polypeptide and the hydrophobic moiety as one contiguous polypeptide chain.

In certain embodiments, the lipophilic moiety is an amphipathic polypeptide, such as magainin, cecropin, attacin, melittin, gramicidin S, alpha-toxin of *Staph. aureus*, alamethicin or a synthetic amphipathic polypeptide. Fusogenic coat proteins from viral particles can also be a convenient source of amphipathic sequences for the subject hedgehog proteins.

Moreover, mutagenesis can be used to create modified *hh* polypeptides, e.g., for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. Modified *hedgehog* polypeptides can also include those with altered post-translational processing relative to a naturally occurring *hedgehog* protein, e.g., altered glycosylation, cholesterolization, prenylation and the like.

In one embodiment, the hedgehog therapeutic is a polypeptide encodable by a nucleotide sequence that hybridizes under stringent conditions to a hedgehog coding sequence represented in one or more of SEQ ID Nos:1-7. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2

x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

As described in the literature, genes for other hedgehog proteins, e.g., from other animals, can be obtained from mRNA or genomic DNA samples using techniques well known in the art. For example, a cDNA encoding a *hedgehog* protein can be obtained by isolating total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding a *hedgehog* protein can also be cloned using established polymerase chain reaction techniques.

Preferred nucleic acids encode a *hedgehog* polypeptide comprising an amino acid sequence at least 60% homologous or identical, more preferably 70% homologous or identical, and most preferably 80% homologous or identical with an amino acid sequence selected from the group consisting of SEQ ID Nos:8-14. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology or identity with an amino acid sequence represented in one of SEQ ID Nos:8-14 are also within the scope of the invention.

In addition to native *hedgehog* proteins, *hedgehog* polypeptides preferred by the present invention are at least 60% homologous or identical, more preferably 70% homologous or identical and most preferably 80% homologous or identical with an amino acid sequence represented by any of SEQ ID Nos:8-14. Polypeptides which are at least 90%, more preferably at least 95%, and most preferably at least about 98-99% homologous or identical with a sequence selected from the group consisting of SEQ ID Nos:8-14 are also within the scope of the invention. The only prerequisite is that the *hedgehog* polypeptide is capable of modulating the growth state of T lymphocytes.

The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a *hedgehog* polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant *hedgehog* gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native *hedgehog* protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

The method of the present invention can also be carried out using variant forms of the naturally occurring *hedgehog* polypeptides, e.g., mutational variants.

As is known in the art, hedgehog polypeptides can be produced by standard biological techniques or by chemical synthesis. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The polypeptide *hedgehog* may be secreted and isolated from a mixture of cells and medium containing the recombinant *hedgehog* polypeptide. Alternatively, the peptide may be retained cytoplasmically by removing the signal peptide sequence from the recombinant *hedgehog* gene and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant *hedgehog* polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant *hedgehog* polypeptide is a fusion protein containing a domain which facilitates its purification, such as an *hedgehog*/GST fusion protein. The host cell may be any prokaryotic or eukaryotic cell.

Recombinant *hedgehog* genes can be produced by ligating nucleic acid encoding an *hedgehog* protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject *hedgehog* polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a *hedgehog* polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due to the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, an *hedgehog* polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of one of the *hedgehog* genes represented in SEQ ID Nos:1-7.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNA1/amp, pcDNA1/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg

derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In some instances, it may be desirable to express the recombinant *hedgehog* polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal containing pBlueBac III).

When it is desirable to express only a portion of an *hedgehog* protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) *PNAS* 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing *hedgehog*-derived polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., *supra*).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. It is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the *hedgehog* polypeptides of the present invention. For example, *hedgehog* polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the *hedgehog* polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence, can be used to

replace the signal sequence which naturally occurs at the N-terminus of the *hedgehog* protein (e.g. of the pro-form, in order to permit purification of the poly(His)-*hedgehog* protein by affinity chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see
5 Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate
10 termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments
15 which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

Hedgehog polypeptides may also be chemically modified to create *hedgehog* derivatives by forming covalent or aggregate conjugates with other chemical moieties, such
20 as glycosyl groups, cholesterol, isoprenoids, lipids, phosphate, acetyl groups and the like. Covalent derivatives of *hedgehog* proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

For instance, *hedgehog* proteins can be generated to include a moiety, other than
25 sequence naturally associated with the protein, that binds a component of the extracellular matrix and enhances localization of the analog to cell surfaces. For example, sequences derived from the fibronectin "type-III repeat", such as a tetrapeptide sequence R-G-D-S (Pierschbacher et al. (1984) *Nature* 309:30-3; and Kornblihtt et al. (1985) *EMBO* 4:1755-9) can be added to the *hedgehog* polypeptide to support attachment of the chimeric molecule
30 to a cell through binding ECM components (Ruoslahti et al. (1987) *Science* 238:491-497; Pierschbacher et al. (1987) *J. Biol. Chem.* 262:17294-8.; Hynes (1987) *Cell* 48:549-54; and Hynes (1992) *Cell* 69:11-25).

In a preferred embodiment, the *hedgehog* polypeptide is isolated from, or is otherwise substantially free of, other cellular proteins, especially other extracellular or cell
35 surface associated proteins which may normally be associated with the *hedgehog* polypeptide, unless provided in the form of fusion protein with the *hedgehog* polypeptide. The term "substantially free of other cellular or extracellular proteins" (also referred to

herein as "contaminating proteins") or "substantially pure preparations" or "purified preparations" are defined as encompassing preparations of *hedgehog* polypeptides having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein. By "purified", it is meant that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in any of SEQ ID Nos:10-18 or 20, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of *hedgehog* polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

With respect to bioactive fragments of *hedgehog* polypeptide, preferred *hedgehog* therapeutics include at least 50 (contiguous) amino acid residues of a *hedgehog* polypeptide, more preferably at least 100 (contiguous), and even more preferably at least 150 (contiguous) residues.

Another preferred *hedgehog* polypeptide which can be included in the *hedgehog* therapeutic is an N-terminal fragment of the mature protein having a molecular weight of approximately 19 kDa.

Preferred human *hedgehog* proteins include N-terminal fragments corresponding approximately to residues 24-197 of SEQ ID No. 15, 28-202 of SEQ ID No. 16, and 23-198 of SEQ ID No. 17. By "corresponding approximately" it is meant that the sequence of interest is at most 20 amino acid residues different in length to the reference sequence, though more preferably at most 5, 10 or 15 amino acid different in length.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in SEQ ID No:8, SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13 or SEQ ID No:14, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of *hedgehog* polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

Still other preferred *hedgehog* polypeptides includes an amino acid sequence represented by the formula A-B wherein: (i) A represents all or the portion of the amino acid sequence designated by residues 1-168 of SEQ ID No:21; and B represents at least one amino acid residue of the amino acid sequence designated by residues 169-221 of SEQ ID No:21; (ii) A represents all or the portion of the amino acid sequence designated by residues 24-193 of SEQ ID No:15; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:15; (iii) A represents all or the portion of the amino acid sequence designated by residues 25-193 of SEQ ID No:13; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:13; (iv) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No:11; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No:11; (v) A represents all or the portion of the amino acid sequence designated by residues 28-197 of SEQ ID No:12; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:12; (vi) A represents all or the portion of the amino acid sequence designated by residues 29-197 of SEQ ID No:16; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID No:16; or (vii) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No. 17, and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No. 17. In certain preferred embodiments, A and B together represent a contiguous polypeptide sequence designated sequence, A represents at least 25, 50, 75, 100, 125 or 150 (contiguous) amino acids of the designated sequence, and B represents at least 5, 10, or 20 (contiguous) amino acid residues of the amino acid sequence designated by corresponding entry in the sequence listing, and A and B together preferably represent a contiguous sequence corresponding to the sequence listing entry. Similar fragments from other *hedgehog* also contemplated, e.g., fragments which correspond to the preferred fragments from the sequence listing entries which are enumerated above. In preferred embodiments, the *hedgehog* polypeptide includes a C-terminal glycine (or other appropriate residue) which is derivatized with a cholesterol.

Isolated peptidyl portions of *hedgehog* proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a *hedgehog* polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl

fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") *hedgehog* protein. For example, Román et al. (1994) *Eur J Biochem* 222:65-73 describe the use of competitive-binding assays using short, overlapping synthetic peptides from larger proteins to identify binding domains.

- 5 The recombinant *hedgehog* polypeptides of the present invention also include homologs of the authentic *hedgehog* proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter potential cleavage sequences or which inactivate an enzymatic activity associated with the protein. *Hedgehog* homologs of the present invention also include proteins which have been post-
- 10 translationally modified in a manner different than the authentic protein. Exemplary derivatives of *hedgehog* proteins include polypeptides which lack N-glycosylation sites (e.g. to produce an unglycosylated protein), which lack sites for cholesterolization, and/or which lack N-terminal and/or C-terminal sequences.

- Modification of the structure of the subject *hedgehog* polypeptides can also be for
- 15 such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the *hedgehog* polypeptides described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution,
- 20 deletion, or addition.

- It is well known in the art that one could reasonably expect that certain isolated replacements of amino acids, e.g., replacement of an amino acid residue with another related amino acid (i.e. isosteric and/or isoelectric mutations), can be carried out without major effect on the biological activity of the resulting molecule. Conservative replacements
- 25 are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In
- 30 similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5)
- 35 amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, *Biochemistry*, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional *hedgehog* homolog (e.g. functional in the sense that it acts to mimic or antagonize the wild-type form)

can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

5 It is specifically contemplated that the methods of the present invention can be carried using homologs of naturally occurring hedgehog proteins. In one embodiment, the invention contemplates using hedgehog polypeptides generated by combinatorial mutagenesis. Such methods, as are known in the art, are convenient for generating both point and truncation mutants, and can be especially useful for identifying potential variant
10 sequences (e.g. homologs) that are functional in binding to a receptor for *hedgehog* proteins. The purpose of screening such combinatorial libraries is to generate, for example, novel *hedgehog* homologs which can act as either agonists or antagonist. To illustrate, *hedgehog* homologs can be engineered by the present method to provide more efficient binding to a cognate receptor, such as *patched*, yet still retain at least a portion of an activity
15 associated with *hedgehog*. Thus, combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein. Likewise, *hedgehog* homologs can be generated by the present combinatorial approach to act as antagonists, in that they are able to mimic, for example, binding to other extracellular matrix components (such as receptors), yet not induce any biological response, thereby
20 inhibiting the action of authentic *hedgehog* or *hedgehog* agonists. Moreover, manipulation of certain domains of *hedgehog* by the present method can provide domains more suitable for use in fusion proteins, such as one that incorporates portions of other proteins which are derived from the extracellular matrix and/or which bind extracellular matrix components.

To further illustrate the state of the art of combinatorial mutagenesis, it is noted that
25 the review article of Gallop et al. (1994) *J Med Chem* 37:1233 describes the general state of the art of combinatorial libraries as of the earlier 1990's. In particular, Gallop et al state at page 1239 "[s]creening the analog libraries aids in determining the minimum size of the active sequence and in identifying those residues critical for binding and intolerant of substitution". In addition, the Ladner et al. PCT publication WO90/02809, the Goeddel et al.
30 al. U.S. Patent 5,223,408, and the Markland et al. PCT publication WO92/15679 illustrate specific techniques which one skilled in the art could utilize to generate libraries of *hedgehog* variants which can be rapidly screened to identify variants/fragments which retained a particular activity of the *hedgehog* polypeptides. These techniques are exemplary of the art and demonstrate that large libraries of related variants/truncants can be generated
35 and assayed to isolate particular variants without undue experimentation. Gustin et al. (1993) *Virology* 193:653, and Bass et al. (1990) *Proteins: Structure, Function and Genetics* 8:309-314 also describe other exemplary techniques from the art which can be adapted as means for generating mutagenic variants of *hedgehog* polypeptides.

Indeed, it is plain from the combinatorial mutagenesis art that large scale mutagenesis of hedgehog proteins, without any preconceived ideas of which residues were critical to the biological function, and generate wide arrays of variants having equivalent biological activity. Indeed, it is the ability of combinatorial techniques to screen billions of
 5 different variants by high throughput analysis that removes any requirement of *a priori* understanding or knowledge of critical residues.

To illustrate, the amino acid sequences for a population of *hedgehog* homologs or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, *hedgehog* homologs from one or
 10 more species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of *hedgehog* variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of
 15 synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *hedgehog* sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of *hedgehog* sequences therein.

As illustrated in PCT publication WO 95/18856, to analyze the sequences of a population of variants, the amino acid sequences of interest can be aligned relative to
 20 sequence homology. The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial.

In an illustrative embodiment, alignment of exons 1, 2 and a portion of exon 3 encoded sequences (e.g. the N-terminal approximately 221 residues of the mature protein)
 25 of each of the *Shh* clones produces a degenerate set of *Shh* polypeptides represented by the general formula:

C-G-P-G-R-G-X(1)-G-X(2)-R-R-H-P-K-K-L-T-P-L-A-Y-K-Q-F-I-P-N-V-
 A-E-K-T-L-G-A-S-G-R-Y-E-G-K-I-X(3)-R-N-S-E-R-F-K-E-L-T-P-N-Y-N-
 P-D-I-I-F-K-D-E-E-N-T-G-A-D-R-L-M-T-Q-R-C-K-D-K-L-N-X(4)-L-A-I-
 30 S-V-M-N-X(5)-W-P-G-V-X(6)-L-R-V-T-E-G-W-D-E-D-G-H-H-X(7)-E-E-
 S-L-H-Y-E-G-R-A-V-D-I-T-T-S-D-R-D-X(8)-S-K-Y-G-X(9)-L-X(10)-R-L-
 A-V-E-A-G-F-D-W-V-Y-Y-E-S-K-A-H-I-H-C-S-V-K-A-E-N-S-V-A-A-K-
 S-G-G-C-F-P-G-S-A-X(11)-V-X(12)-L-X(13)-X(14)-G-G-X(15)-K-X(16)-
 V-K-D-L-X(17)-P-G-D-X(18)-V-L-A-A-D-X(19)-X(20)-G-X(21)-L-X(22)-
 35 X(23)-S-D-F-X(24)-X(25)-F-X(26)-D-R (SEQ ID No: 21)

wherein each of the degenerate positions "X" can be an amino acid which occurs in that position in one of the human, mouse, chicken or zebrafish *Shh* clones, or, to expand the library, each X can also be selected from amongst amino acid residue which would be conservative substitutions for the amino acids which appear naturally in each of those

positions. For instance, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Phe, Tyr or Trp ; Xaa(2) represents Arg, His or Lys; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(4) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(5) represents Lys, Arg, His, Asn or Gln; Xaa(6) represents Lys, Arg or His; Xaa(7) represents Ser, Thr, Tyr, Trp or Phe; Xaa(8) represents Lys, Arg or His; Xaa(9) represents Met, Cys, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Leu, Val, Met, Thr or Ser; Xaa(12) represents His, Phe, Tyr, Ser, Thr, Met or Cys; Xaa(13) represents Gln, Asn, Glu, or Asp; Xaa(14) represents His, Phe, Tyr, Thr, Gln, Asn, Glu or Asp; Xaa(15) represents Gln, Asn, Glu, Asp, Thr, Ser, Met or Cys; Xaa(16) represents Ala, Gly, Cys, Leu, Val or Met; Xaa(17) represents Arg, Lys, Met, Ile, Asn, Asp, Glu, Gln, Ser, Thr or Cys; Xaa(18) represents Arg, Lys, Met or Ile; Xaa(19) represents Ala, Gly, Cys, Asp, Glu, Gln, Asn, Ser, Thr or Met; Xaa(20) represents Ala, Gly, Cys, Asp, Asn, Glu or Gln; Xaa(21) represents Arg, Lys, Met, Ile, Asn, Asp, Glu or Gln; Xaa(22) represent Leu, Val, Met or Ile; Xaa(23) represents Phe, Tyr, Thr, His or Trp; Xaa(24) represents Ile, Val, Leu or Met; Xaa(25) represents Met, Cys, Ile, Leu, Val, Thr or Ser; Xaa(26) represents Leu, Val, Met, Thr or Ser. In an even more expansive library, each X can be selected from any amino acid.

In similar fashion, alignment of each of the human, mouse, chicken and zebrafish *hedgehog* clones, can provide a degenerate polypeptide sequence represented by the general formula:

20 C-G-P-G-R-G-X(1)-X(2)-X(3)-R-R-X(4)-X(5)-X(6)-P-K-X(7)-L-X(8)-P-L-
X(9)-Y-K-Q-F-X(10)-P-X(11)-X(12)-X(13)-E-X(14)-T-L-G-A-S-G-X(15)-
X(16)-E-G-X(17)-X(18)-X(19)-R-X(20)-S-E-R-F-X(21)-X(22)-L-T-P-N-Y-
N-P-D-I-I-F-K-D-E-E-N-X(23)-G-A-D-R-L-M-T-X(24)-R-C-K-X(25)-
25 X(26)-X(27)-N-X(28)-L-A-I-S-V-M-N-X(29)-W-P-G-V-X(30)-L-R-V-T-E-
G-X(31)-D-E-D-G-H-H-X(32)-X(33)-X(34)-S-L-H-Y-E-G-R-A-X(35)-D-I-
T-T-S-D-R-D-X(36)-X(37)-K-Y-G-X(38)-L-X(39)-R-L-A-V-E-A-G-F-D-
W-V-Y-Y-E-S-X(40)-X(41)-H-X(42)-H-X(43)-S-V-K-X(44)-X(45) (SEQ
IDNo:22

wherein, as above, each of the degenerate positions "X" can be an amino acid which occurs in a corresponding position in one of the wild-type clones, and may also include amino acid residue which would be conservative substitutions, or each X can be any amino acid residue. In an exemplary embodiment, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Pro, Phe or Tyr; Xaa(2) represents Gly, Ala, Val, Leu or Ile; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Lys, His or Arg; Xaa(4) represents Lys, Arg or His; Xaa(5) represents Phe, Trp, Tyr or an amino acid gap; Xaa(6) represents Gly, Ala, Val, Leu, Ile or an amino acid gap; Xaa(7) represents Asn, Gln, His, Arg or Lys; Xaa(8) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(9) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Ser, Thr, Gln or Asn; Xaa(12) represents Met, Cys, Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(13) represents Gly, Ala, Val, Leu, Ile or Pro; Xaa(14) represents Arg, His or Lys; Xaa(15) represents Gly, Ala, Val, Leu, Ile, Pro, Arg, His or Lys;

Xaa(16) represents Gly, Ala, Val, Leu, Ile, Phe or Tyr; Xaa(17) represents Arg, His or Lys; Xaa(18) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(19) represents Thr or Ser; Xaa(20) represents Gly, Ala, Val, Leu, Ile, Asn or Gln; Xaa(21) represents Arg, His or Lys; Xaa(22) represents Asp or Glu; Xaa(23) represents Ser or Thr; Xaa(24) represents Glu, Asp, Gln or Asn; Xaa(25) represents Glu or Asp; Xaa(26) represents Arg, His or Lys; Xaa(27) represents Gly, Ala, Val, Leu or Ile; Xaa(28) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(29) represents Met, Cys, Gln, Asn, Arg, Lys or His; Xaa(30) represents Arg, His or Lys; Xaa(31) represents Trp, Phe, Tyr, Arg, His or Lys; Xaa(32) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Tyr or Phe; Xaa(33) represents Gln, Asn, Asp or Glu; Xaa(34) represents Asp or Glu; Xaa(35) represents Gly, Ala, Val, Leu, or Ile; Xaa(36) represents Arg, His or Lys; Xaa(37) represents Asn, Gln, Thr or Ser; Xaa(38) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Met or Cys; Xaa(39) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(40) represents Arg, His or Lys; Xaa(41) represents Asn, Gln, Gly, Ala, Val, Leu or Ile; Xaa(42) represents Gly, Ala, Val, Leu or Ile; Xaa(43) represents Gly, Ala, Val, Leu, Ile, Ser, Thr or Cys; Xaa(44) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; and Xaa(45) represents Asp or Glu.

There are many ways by which the library of potential *hedgehog* homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *hedgehog* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249:404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of *hedgehog* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy

isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate *hedgehog* sequences created by combinatorial mutagenesis techniques.

- 5 In one embodiment, the combinatorial library is designed to be secreted (e.g. the polypeptides of the library all include a signal sequence but no transmembrane or cytoplasmic domains), and is used to transfect a eukaryotic cell that can be co-cultured with peripheal nerve cells. A functional *hedgehog* protein secreted by the cells expressing the combinatorial library will diffuse to neighboring T lymphocytes and induce a particular
10 biological response, such as proliferation or differentiation. The pattern of detection of such a change in phenotype will resemble a gradient function, and will allow the isolation (generally after several repetitive rounds of selection) of cells producing *hedgehog* homologs active as neurotrophic agents. Likewise, *hedgehog* antagonists can be selected in similar fashion by the ability of the cell producing a functional antagonist to protect
15 neighboring cells (e.g., to inhibit proliferation) from the effect of wild-type *hedgehog* added to the culture media.

- To illustrate, target T lymphocytes are cultured in 24-well microtitre plates. Other eukaryotic cells are transfected with the combinatorial *hedgehog* gene library and cultured in cell culture inserts (e.g. Collaborative Biomedical Products, Catalog #40446) that are
20 able to fit into the wells of the microtitre plate. The cell culture inserts are placed in the wells such that recombinant *hedgehog* homologs secreted by the cells in the insert can diffuse through the porous bottom of the insert and contact the target cells in the microtitre plate wells. After a period of time sufficient for functional forms of a *hedgehog* protein to produce a measurable response in the target cells, such as growth state, the inserts are
25 removed and the effect of the variant *hedgehog* proteins on the target cells determined. Cells from the inserts corresponding to wells which score positive for activity can be split and re-cultured on several inserts, the process being repeated until the active clones are identified.

- In yet another screening assay, the candidate *hedgehog* gene products are displayed
30 on the surface of a cell or viral particle, and the ability of particular cells or viral particles to associate with a *hedgehog*-binding moiety (such as the *patched* protein or other *hedgehog* receptor) via this gene product is detected in a "panning assay". Such panning steps can be carried out on cells cultured from embryos. For instance, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion
35 protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, fluorescently labeled molecules which bind *hedgehog* can be used to score for potentially functional *hedgehog* homologs. Cells can be visually inspected and separated

under a fluorescence microscope, or, where the morphology of the cell permits, separated by a fluorescence-activated cell sorter.

In an alternate embodiment, the gene library is expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E.coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) J. Biol. Chem. 267:16007-16010; Griffiths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457-4461).

In an illustrative embodiment, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing and screening *hedgehog* combinatorial libraries. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The *hedgehog* combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate *hedgehog* gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate *hedgehog*, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate *hedgehog* proteins which are capable of binding an *hedgehog* receptor are selected or enriched by panning. For instance, the phage library can be applied to cells which express the *patched* protein and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for *hedgehog* homologs, which can then be screened for further biological activities in order to differentiate agonists and antagonists.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of 10^{26} molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays such as phage display. To overcome this problem, a new technique has been developed recently, recursive

ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, *PNAS USA* 89:7811-7815; Yourvan et al., 1992, *Parallel Problem Solving from Nature*, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, *Protein Engineering* 6(3):327-331).

The invention also provides for reduction of the *hedgehog* protein to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt binding of a *hedgehog* polypeptide of the present invention with an *hedgehog* receptor. Thus, such mutagenic techniques as described above are also useful to map the determinants of the *hedgehog* proteins which participate in protein-protein interactions involved in, for example, binding of the subject *hedgehog* polypeptide to other extracellular matrix components. To illustrate, the critical residues of a subject *hedgehog* polypeptide which are involved in molecular recognition of an *hedgehog* receptor such as *patched* can be determined and used to generate *hedgehog*-derived peptidomimetics which competitively inhibit binding of the authentic *hedgehog* protein with that moiety. By employing, for example, scanning mutagenesis to map the amino acid residues of each of the subject *hedgehog* proteins which are involved in binding other extracellular proteins, peptidomimetic compounds can be generated which mimic those residues of the *hedgehog* protein which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of a *hedgehog* protein. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β -turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β -aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Recombinantly produced forms of the *hedgehog* proteins can be produced using, e.g. expression vectors containing a nucleic acid encoding a *hedgehog* polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is

intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of a *hedgehog* polypeptide. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding *hedgehog* polypeptide. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

In addition to providing a ready source of *hedgehog* polypeptides for purification, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of a *hedgehog* polypeptide. Thus, another aspect of the invention features expression vectors for *in vivo* transfection of a *hedgehog* polypeptide in particular cell types so as cause ectopic expression of a *hedgehog* polypeptide in an T lymphocytes or other cells associated therewith.

Formulations of such expression constructs may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the *hedgehog* coding sequence in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular

carriers, as well as direct injection of the gene construct or CaPO₄ precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of *hedgehog* expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture systems described below.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the particular form of the *hedgehog* polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding a *hedgehog* polypeptide and renders the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include Crip, Cre, 2 and Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including T lymphocytes, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc.*

- Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644;
- 5 Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and WO94/06920). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julan et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the *hedgehog* gene of the retroviral vector.

Another viral gene delivery system useful in the present method utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155.

30 Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they can be used to infect a wide variety of cell types, including T lymphocytes. Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above,

35 can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated

into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore
5 favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted *hedgehog* gene can be under control of, for example, the E1A promoter, the major late
10 promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a *hedgehog* polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by
15 mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the *hedgehog* polypeptide gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In clinical settings, the gene delivery systems for the therapeutic *hedgehog* gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be
20 introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle
25 can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057). A *hedgehog* expression construct can be delivered in a gene therapy construct to dermal cells by, e.g., electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix
35 in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In yet another embodiment, the hedgehog or *ptc* therapeutic can be a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous gene. For instance, the gene activation construct can replace the endogenous promoter of a *hedgehog* gene with a
5 heterologous promoter, e.g., one which causes constitutive expression of the *hedgehog* gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. Other genes in the *patched* signaling pathway can be similarly targeted. A variety of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications
10 WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous *hedgehog* gene (exon sequence, intron sequence, promoter sequences, etc.) which direct
15 recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic *hedgehog* gene upon recombination of the gene activation construct. For use in generating cultures of *hedgehog* producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic
20 DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native *hedgehog* gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous *hedgehog* gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding
25 position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking
30 sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement
35 region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof. Promoters/enhancers

which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human β -actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

In an exemplary embodiment, portions of the 5' flanking region of the human Shh gene are amplified using primers which add restriction sites, to generate the following fragments

5' -gcgcgcttcgaaGCGAGGCAGCCAGCGAGGGAGAGAGCGAGCGGGCGAGCCGGAGC-GAGGAAatcgatgcgcgcg (primer 1)

5' -gcgcgcgagatctGGGAAAGCGCAAGAGAGAGCGCACACGCACACACCCGCCGCGCG-CACTCGggatccgcgcgcg (primer 2)

As illustrated, primer 1 includes a 5' non-coding region of the human Shh gene and is flanked by an *AsuII* and *ClaI* restriction sites. Primer 2 includes a portion of the 5' non-coding region immediately 3' to that present in primer 1. The hedgehog gene sequence is flanked by *XhoII* and *BamHI* restriction sites. The purified amplimers are cut with each of the enzymes as appropriate.

The vector pCDNA1.1 (Invitrogen) includes a CMV promoter. The plasmid is cut with *AsuII*, which cleaves just 3' to the CMV promoter sequence. The *AsuII/ClaI* fragment of primer 1 is ligated to the *AsuII* cleavage site of the pCDNA vector. The *ClaI/AsuII* ligation destroys the *AsuII* site at the 3' end of a properly inserted primer 1.

The vector is then cut with *BamHI*, and an *XhoII/BamHI* fragment of primer 2 is ligated to the *BamHI* cleavage site. As above, the *BamHI/XhoII* ligation destroys the *BamHI* site at the 5' end of a properly inserted primer 2.

Individual colonies are selected, cut with *AsuII* and *BamHI*, and the size of the *AsuII/BamHI* fragment determined. Colonies in which both the primer 1 and primer 2

sequences are correctly inserted are further amplified, an cut with *AsuII* and *BamHI* to produce the gene activation construct

cgaagcgaggcagccagcgagggagagagcgagcgggagcgaggagcgaggaaATCGAAG
 GTTCGAATCCTTCCCCACCACCATCACTTTCAAAGTCCGAAAGAATCTGCTCCCTGCTT
 5 GTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGTAAATTTAAGCTACAACAAGGCAAGGCTT
 GACCGACAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTA
 CGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGG
 GGTCATTAGTTCATAGCCCATATATGGAGTTCGCGGTTACATAACTTACGGTAAATGGCCC
 GCCTGGCTGACCGCCCAACGACCCCGCCCATTTGACGTCAATAATGACGTATGTTCCCATTA
 10 GTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCC
 ACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGG
 TAAATGGCCCGCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCTTACTTGGCAG
 TACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATG
 GGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTTGACGTCAATGG
 15 GAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTGTAACAACCTCCGCCCCA
 TTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGC
 TAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGAC
 CCAAGCTTGGTACCGAGCTCGGATCgatctgggaaagcgcaagagagagcgcacacgcaca
 caccgcgcgcgcactcgg

20 In this construct, the flanking primer 1 and primer 2 sequences provide the recombination region which permits the insertion of the CMV promoter in front of the coding sequence for the human *Shh* gene. Other heterologous promoters (or other transcriptional regulatory sequences) can be inserted in a genomic *hedgehog* gene by a similar method.

In still other embodiments, the replacement region merely deletes a negative
 25 transcriptional control element of the native gene, e.g., to activate expression, or ablates a positive control element, e.g., to inhibit expression of the targeted gene.

V. Exemplary ptc therapeutic compounds.

In another embodiment, the subject method is carried out using a ptc therapeutic
 30 composition. Such compositions can be generated with, for example, compounds which bind to patched and alter its signal transduction activity, compounds which alter the binding and/or enzymatic activity of a protein (e.g., intracellular) involved in patched signal pathway, and compounds which alter the level of expression of a hedgehog protein, a patched protein or a protein involved in the intracellular signal transduction pathway of
 35 patched.

The availability of purified and recombinant *hedgehog* polypeptides facilitates the generation of assay systems which can be used to screen for drugs, such as small organic molecules, which are either agonists or antagonists of the normal cellular function of a *hedgehog* and/or *patched* protein, particularly their role in the pathogenesis of peripheral nerve proliferation and/or differentiation. In one embodiment, the assay evaluates the ability of a compound to modulate binding between a *hedgehog* polypeptide and a *hedgehog* receptor such as *patched*. In other embodiments, the assay merely scores for the ability of a test compound to alter the signal transduction activity of the *patched* protein. In this manner, a variety of *hedgehog* and/or *ptc* therapeutics, both agonists (inhibit T-cell maturation) and antagonist (promote T-cell maturation) can be identified. A variety of assay formats will suffice and, in light of the present disclosure, will be comprehended by skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with receptor proteins.

Accordingly, in an exemplary screening assay for *ptc* therapeutics, the compound of interest is contacted with a mixture including a *hedgehog* receptor protein (e.g., a cell expressing the *patched* receptor) and a *hedgehog* protein under conditions in which it is ordinarily capable of binding the *hedgehog* protein. To the mixture is then added a composition containing a test compound. Detection and quantification of receptor/*hedgehog* complexes provides a means for determining the test compound's efficacy at inhibiting (or potentiating) complex formation between the receptor protein and the *hedgehog* polypeptide. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified *hedgehog* polypeptide is added to the receptor protein, and the formation of receptor/*hedgehog* complex is quantitated in the absence of the test compound.

In other embodiments, a *ptc* therapeutic of the present invention is one which disrupts the association of *patched* with *smoothened*.

Agonist and antagonists of peripheral nerve maintenance can be distinguished, and the efficacy of the compound can be assessed, by subsequent testing with T lymphocytes, e.g., in culture.

In an illustrative embodiment, the polypeptide utilized as a *hedgehog* receptor can be generated from the *patched* protein. Accordingly, an exemplary screening assay includes all or a suitable portion of the *patched* protein which can be obtained from, for example, the human *patched* gene (GenBank U43148) or other vertebrate sources (see GenBank Accession numbers U40074 for chicken *patched* and U46155 for mouse *patched*), as well as from drosophila (GenBank Accession number M28999) or other invertebrate sources. The *patched* protein can be provided in the screening assay as a whole protein (preferably expressed on the surface of a cell), or alternatively as a fragment of the full length protein which binds to *hedgehog* polypeptides, e.g., as one or both of the substantial extracellular domains (e.g. corresponding to residues Asn120-Ser438 and/or Arg770-Trp1027 of the human *patched* protein - which are also potential antagonists of *hedgehog*-dependent signal transduction). For instance, the *patched* protein can be provided in soluble form, as for example a preparation of one of the extracellular domains, or a preparation of both of the extracellular domains which are covalently connected by an unstructured linker (see, for example, Huston et al. (1988) PNAS 85:4879; and U.S. Patent No. 5,091,513). In other embodiments, the protein can be provided as part of a liposomal preparation or expressed on the surface of a cell. The *patched* protein can be derived from a recombinant gene, e.g., being ectopically expressed in a heterologous cell. For instance, the protein can be expressed on oocytes, mammalian cells (e.g., COS, CHO, 3T3 or the like), or yeast cell by standard recombinant DNA techniques. These recombinant cells can be used for receptor binding, signal transduction or gene expression assays. Marigo et al. (1996) *Development* 122:1225-1233 illustrates a binding assay of human *hedgehog* to chick *patched* protein ectopically expressed in *Xenopus laevis* oocytes. The assay system of Marigo et al. can be adapted to the present drug screening assays. As illustrated in that reference, *Shh* binds to the *patched* protein in a selective, saturable, dose-dependent manner, thus demonstrating that *patched* is a receptor for *Shh*.

Complex formation between the *hedgehog* polypeptide and a *hedgehog* receptor may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labelled proteins such as radiolabelled, fluorescently labelled, or enzymatically labelled *hedgehog* polypeptides, by immunoassay, or by chromatographic detection.

Typically, for cell-free assays, it will be desirable to immobilize either the *hedgehog* receptor or the *hedgehog* polypeptide to facilitate separation of receptor/*hedgehog* complexes from uncomplexed forms of one of the proteins, as well as to accommodate

automation of the assay. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/receptor (GST/receptor) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre
5 plates, which are then combined with the *hedgehog* polypeptide, e.g. an ^{35}S -labeled *hedgehog* polypeptide, and the test compound and incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound *hedgehog* polypeptide, and the matrix bead-bound radiolabel determined
10 directly (e.g. beads placed in scintillant), or in the supernatant after the receptor/*hedgehog* complexes are dissociated. Alternatively, the complexes can be dissociated from the bead, separated by SDS-PAGE gel, and the level of *hedgehog* polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in
15 the subject assay. For instance, soluble portions of the *hedgehog* receptor protein can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated receptor molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical).
20 Alternatively, antibodies reactive with the *hedgehog* receptor but which do not interfere with *hedgehog* binding can be derivatized to the wells of the plate, and the receptor trapped in the wells by antibody conjugation. As above, preparations of a *hedgehog* polypeptide and a test compound are incubated in the receptor-presenting wells of the plate, and the amount of receptor/*hedgehog* complex trapped in the well can be quantitated. Exemplary
25 methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the *hedgehog* polypeptide, or which are reactive with the receptor protein and compete for binding with the *hedgehog* polypeptide; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the *hedgehog* polypeptide. In the instance
30 of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the *hedgehog* polypeptide. To illustrate, the *hedgehog* polypeptide can be chemically cross-linked or genetically fused with alkaline phosphatase, and the amount of *hedgehog* polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. paranitrophenylphosphate. Likewise, a fusion protein comprising the
35 *hedgehog* polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) *J Biol Chem* 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as the anti-*hedgehog* antibodies described herein, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the *hedgehog* polypeptide or *hedgehog* receptor sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, NJ).

Where the desired portion of the *hedgehog* receptor (or other *hedgehog* binding molecule) cannot be provided in soluble form, liposomal vesicles can be used to provide manipulatable and isolatable sources of the receptor. For example, both authentic and recombinant forms of the *patched* protein can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374).

In addition to cell-free assays, such as described above, the readily available source of *hedgehog* proteins provided by the art also facilitates the generation of cell-based assays for identifying small molecule agonists/antagonists and the like. Analogous to the cell-based assays described above for screening combinatorial libraries, cells which are sensitive to *hedgehog* induction, e.g. *patched*-expressing cells or other myoblast-derived cells sensitive to *hedgehog* induction, can be contacted with a *hedgehog* protein and a test agent of interest, with the assay scoring for anything from simple binding to the cell to modulation in *hedgehog* inductive responses by the target cell in the presence and absence of the test agent. As with the cell-free assays, agents which produce a statistically significant change in *hedgehog* activities (either inhibition or potentiation) can be identified.

In other embodiments, the cell-based assay scores for agents which disrupt association of *patched* and *smoothed* proteins, e.g., in the cell surface membrane or liposomal preparation.

In addition to characterizing cells that naturally express the *patched* protein, cells which have been genetically engineered to ectopically express *patched* can be utilized for drug screening assays. As an example, cells which either express low levels or lack expression of the *patched* protein, e.g. *Xenopus laevis* oocytes, COS cells or yeast cells, can

be genetically modified using standard techniques to ectopically express the *patched* protein. (see Marigo et al., *supra*).

The resulting recombinant cells, e.g., which express a functional *patched* receptor, can be utilized in receptor binding assays to identify agonist or antagonists of *hedgehog* binding. Binding assays can be performed using whole cells. Furthermore, the recombinant cells of the present invention can be engineered to include other heterologous genes encoding proteins involved in *hedgehog*-dependent signal pathways. For example, the gene products of one or more of *smoothened*, *costal-2* and/or *fused* can be co-expressed with *patched* in the reagent cell, with assays being sensitive to the functional reconstitution of the *hedgehog* signal transduction cascade.

Alternatively, liposomal preparations using reconstituted *patched* protein can be utilized. *Patched* protein purified from detergent extracts from both authentic and recombinant origins can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374). The lamellar structure and size of the resulting liposomes can be characterized using electron microscopy. External orientation of the *patched* protein in the reconstituted membranes can be demonstrated, for example, by immunoelectron microscopy. The *hedgehog* protein binding activity of liposomes containing *patched* and liposomes without the protein in the presence of candidate agents can be compared in order to identify potential modulators of the *hedgehog-patched* interaction.

The *hedgehog* protein used in these cell-based assays can be provided as a purified source (natural or recombinant in origin), or in the form of cells/tissue which express the protein and which are co-cultured with the target cells. As in the cell-free assays, where simple binding (rather than induction) is the *hedgehog* activity scored for in the assay, the protein can be labelled by any of the above-mentioned techniques, e.g., fluorescently, enzymatically or radioactively, or detected by immunoassay.

In addition to binding studies, functional assays can be used to identify modulators, i.e., agonists or antagonists, of *hedgehog* or *patched* activities. By detecting changes in intracellular signals, such as alterations in second messengers or gene expression, in *patched*-expressing cells contacted with a test agent, candidate agonists and antagonists to *patched* signaling can be identified.

A number of gene products have been implicated in *patched*-mediated signal transduction, including *patched*, the transcription factor *cubitus interruptus* (ci), the serine/threonine kinase *fused* (fu) and the gene products of *costal-2*, *smoothened* and *suppressor of fused*.

The interaction of a hedgehog protein with *patched* sets in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or translation of a gene. Potential transcriptional targets of *patched* signaling are the *patched* gene itself (Hidalgo and Ingham, 1990 *Development* 110, 291-301; Marigo et al., 1996) and the vertebrate homologs of the drosophila cubitus interruptus gene, the *GLI* genes (Hui et al. (1994) *Dev Biol* 162:402-413). *Patched* gene expression has been shown to be induced in cells of the limb bud and the neural plate that are responsive to *Shh*. (Marigo et al. (1996) *PNAS*, in press; Marigo et al. (1996) *Development* 122:1225-1233). The *GLI* genes encode putative transcription factors having zinc finger DNA binding domains (Orenic et al. (1990) *Genes & Dev* 4:1053-1067; Kinzler et al. (1990) *Mol Cell Biol* 10:634-642). Transcription of the *GLI* gene has been reported to be upregulated in response to *hedgehog* in limb buds, while transcription of the *GLI3* gene is downregulated in response to *hedgehog* induction (Marigo et al. (1996) *Development* 122:1225-1233). By selecting transcriptional regulatory sequences from such target genes, e.g. from *patched* or *GLI* genes, that are responsible for the up- or down regulation of these genes in response to *patched* signalling, and operatively linking such promoters to a reporter gene, one can derive a transcription based assay which is sensitive to the ability of a specific test compound to modify *patched* signalling pathways. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as agonists or antagonists of *ptc* induction of differentiation/quiescence.

Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on *ptc* signaling. To identify potential regulatory elements responsive to *ptc* signaling present in the transcriptional regulatory sequence of a target gene, nested deletions of genomic clones of the target gene can be constructed using standard techniques. See, for example, Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989); U.S. Patent 5,266,488; Sato et al. (1995) *J Biol Chem* 270:10314-10322; and Kube et al. (1995) *Cytokine* 7:1-7. A nested set of DNA fragments from the gene's 5'-flanking region are placed upstream of a reporter gene, such as the luciferase gene, and assayed for their ability to direct reporter gene expression in *patched* expressing cells. Host cells transiently transfected with reporter gene constructs can be scored for the induction of expression of the reporter gene in the presence and absence of *hedgehog* to determine regulatory sequences which are responsive to *patched*-dependent signalling.

In practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on second messengers

generated by induction with *hedgehog* protein. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the *hedgehog* activity, with the level of expression of the reporter gene providing the *hedgehog*-dependent detection signal. The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNase protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound (or *hedgehog*) or it may be compared with the amount of transcription in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the signal transduction of the *patched* protein, e.g., the test compound is a potential *ptc* therapeutic.

As described in further detail below, in preferred embodiments the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence. In other preferred embodiments, the reporter or marker gene provides a selective growth advantage, e.g., the reporter gene may enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug.

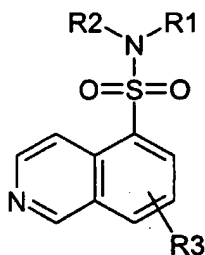
Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), *Nature* 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), *Mol. Cell. Biol.* 7:725-737); bacterial luciferase (Engbrecht and Silverman (1984), *PNAS* 1: 4154-4158; Baldwin et al. (1984), *Biochemistry* 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) *Eur. J. Biochem.* 182: 231-238, Hall et al. (1983) *J. Mol. Appl. Gen.* 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) *Methods in Enzymol.* 216:362-368).

Transcriptional control elements which may be included in a reporter gene construct include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is induced after modulation of a *patched* signal transduction pathway. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or

undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular simulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

In yet other embodiments, second messenger generation can be measured directly in the detection step, such as mobilization of intracellular calcium, phospholipid metabolism or adenylate cyclase activity are quantitated, for instance, the products of phospholipid hydrolysis IP₃, DAG or cAMP could be measured. For example, recent studies have implicated protein kinase A (PKA) as a possible component of *hedgehog/patched* signaling (Hammerschmidt et al. (1996) *Genes & Dev* 10:647). High PKA activity has been shown to antagonize *hedgehog* signaling in these systems. Although it is unclear whether PKA acts directly downstream or in parallel with *hedgehog* signaling, it is possible that *hedgehog* signalling occurs via inhibition of PKA activity. Thus, detection of PKA activity provides a potential readout for the instant assays.

In a preferred embodiment, the *ptc* therapeutic is a PKA inhibitor. A variety of PKA inhibitors are known in the art, including both peptidyl and organic compounds. For instance, the *ptc* therapeutic can be a 5-isoquinolinesulfonamide, such as represented in the general formula:



wherein,

R₁ and R₂ each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, -(CH₂)_m-R₈, -(CH₂)_m-OH, -(CH₂)_m-O-lower alkyl, -(CH₂)_m-O-lower alkenyl, -(CH₂)_n-O-(CH₂)_m-R₈, -(CH₂)_m-SH, -(CH₂)_m-S-lower alkyl, -(CH₂)_m-S-lower alkenyl, -(CH₂)_n-S-(CH₂)_m-R₈, or

R₁ and R₂ taken together with N form a heterocycle (substituted or unsubstituted);

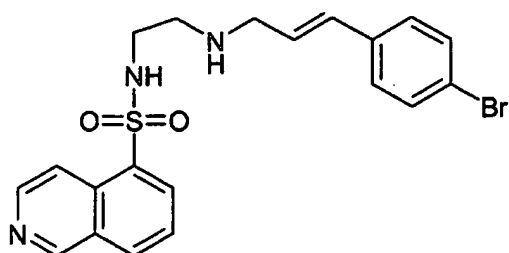
R₃ is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a

- formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

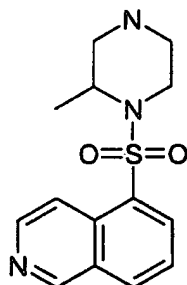
R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

- 10 In a preferred embodiment, the PKA inhibitor is N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89; Calbiochem Cat. No. 371963), e.g., having the formula:

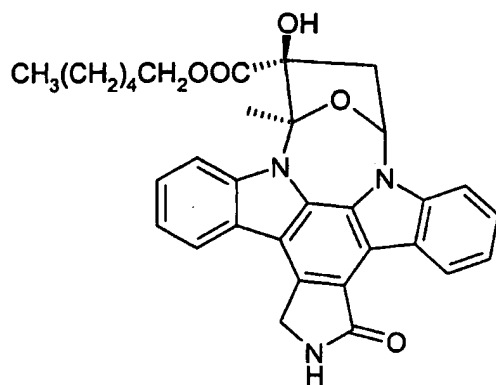


In another embodiment, the PKA inhibitor is 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7; Calbiochem Cat. No. 371955), e.g., having the formula:

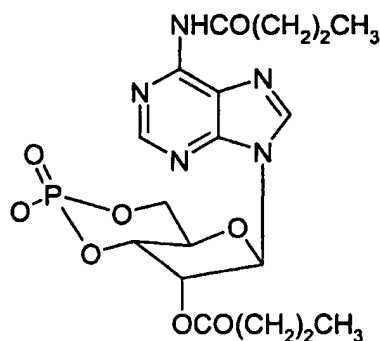
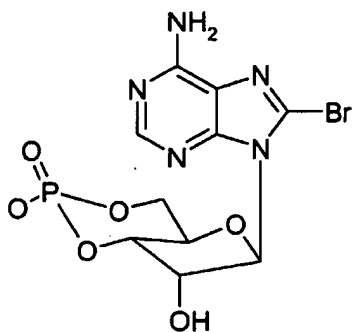


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In still other embodiments, the PKA inhibitor is KT5720 (Calbiochem Cat. No. 420315), having the structure



A variety of nucleoside analogs are also useful as PKA inhibitors. For example, the subject method can be carried out cyclic AMP analogs which inhibit the kinase activity of PKA, as for example, 8-bromo-cAMP or dibutyl-cAMP



5

Exemplary peptidyl inhibitors of PKA activity include the PKA Heat Stable Inhibitor (isoform α ; see, for example, Calbiochem Cat. No. 539488, and Wen et al. (1995) *J Biol Chem* 270:2041).

Certain *hedgehog* receptors may stimulate the activity of phospholipases. Inositol lipids can be extracted and analyzed using standard lipid extraction techniques. Water soluble derivatives of all three inositol lipids (IP₁, IP₂, IP₃) can also be quantitated using radiolabelling techniques or HPLC.

The mobilization of intracellular calcium or the influx of calcium from outside the cell may be a response to *hedgehog* stimulation or lack thereof. Calcium flux in the reagent cell can be measured using standard techniques. The choice of the appropriate calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca⁺⁺-sensitive microelectrodes depends on the cell type and the magnitude and time constant of the event under study (Borle (1990) *Environ Health Perspect* 84:45-56). As an exemplary method of Ca⁺⁺ detection, cells could be loaded with the Ca⁺⁺-sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in Ca⁺⁺ measured using a fluorometer.

20

In certain embodiments of the assay, it may be desirable to screen for changes in cellular phosphorylation. As an example, the drosophila gene *fused* (*fu*) which encodes a serine/threonine kinase has been identified as a potential downstream target in *hedgehog* signaling. (Preat et al., 1990 *Nature* 347, 87-89; Therond et al. 1993, *Mech. Dev.* 44. 65-80). The ability of compounds to modulate serine/threonine kinase activation could be screened using colony immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430) using antibodies against phosphorylated serine or threonine residues. Reagents for performing such assays are commercially available, for example, phosphoserine and phosphothreonine specific antibodies which measure increases in phosphorylation of those residues can be purchased from commercial sources.

In yet another embodiment, the *ptc* therapeutic is an antisense molecule which inhibits expression of a protein involved in a *patched*-mediated signal transduction pathway. To illustrate, by inhibiting the expression of a protein which are involved in *patched* signals, such as *fused*, *costal-2*, *smoothened* and/or *Gli* genes, the ability of the *patched* signal pathway(s) to inhibit proliferation of a cell can be altered, e.g., potentiated or repressed.

As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions with cellular mRNA and/or genomic DNA encoding a *hedgehog* protein, *patched*, or a protein involved in *patched*-mediated signal transduction. The hybridization should inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the target cellular mRNA. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a target gene. Such oligonucleotide probes are preferably modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense

therapy have been reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

Several considerations should be taken into account when constructing antisense oligonucleotides for the use in the methods of the invention: (1) oligos should have a GC
 5 content of 50% or more; (2) avoid sequences with stretches of 3 or more G's; and (3) oligonucleotides should not be longer than 25-26 mers. When testing an antisense oligonucleotide, a mismatched control can be constructed. The controls can be generated by reversing the sequence order of the corresponding antisense oligonucleotide in order to conserve the same ratio of bases.

10 In an illustrative embodiment, the *ptc* therapeutic can be an antisense construct for inhibiting the expression of *patched*, e.g., to mimic the inhibition of *patched* by *hedgehog*. Exemplary antisense constructs include:

5'-GTCCTGGCGCCGCCGCCGCCGTCGCC

5'-TTCCGATGACCGGCCTTTCGCGGTGA

15 5'-GTGCACGGAAAGGTGCAGGCCACACT

VI. Exemplary pharmaceutical preparations of hedgehog and ptc therapeutics

The source of the hedgehog and *ptc* therapeutics to be formulated will depend on the particular form of the agent. Small organic molecules and peptidyl fragments can be
 20 chemically synthesized and provided in a pure form suitable for pharmaceutical/cosmetic usage. Products of natural extracts can be purified according to techniques known in the art. For example, the Cox et al. U.S. Patent 5,286,654 describes a method for purifying naturally occurring forms of a secreted protein and can be adapted for purification of hedgehog polypeptides. Recombinant sources of hedgehog polypeptides are also available.
 25 For example, the gene encoding *hedgehog* polypeptides, are known, *inter alia*, from PCT publications WO 95/18856 and WO 96/17924.

Those of skill in treating peripheral neuropathies can determine the effective amount of an hedgehog or *ptc* therapeutic to be formulated in a pharmaceutical or cosmetic preparation.

30 The hedgehog or *ptc* therapeutic formulations used in the method of the invention are most preferably applied in the form of appropriate compositions. As appropriate compositions there may be cited all compositions usually employed for systemically or topically administering drugs. The pharmaceutically acceptable carrier should be substantially inert, so as not to act with the active component. Suitable inert carriers include

water, alcohol polyethylene glycol, mineral oil or petroleum gel, propylene glycol and the like.

To prepare the pharmaceutical compositions of this invention, an effective amount of the particular hedgehog or ptc therapeutic as the active ingredient is combined in intimate admixture with a pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical compositions are desirable in unitary dosage form suitable, particularly, for administration orally, rectally, percutaneously, or by parenteral injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in the case of powders, pills, capsules, and tablets. Because of their ease in administration, tablets and capsules represents the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations. In the compositions suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a suitable wetting agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not introduce a significant deleterious effect on the skin.

In addition to the direct topical application of the preparations they can be topically administered by other methods, for example, encapsulated in a temperature and/or pressure sensitive matrix or in film or solid carrier which is soluble in body fluids and the like for subsequent release, preferably sustained-release of the active component.

As appropriate compositions for topical application there may be cited all compositions usually employed for topically administering therapeutics, e.g., creams, gellies, dressings, shampoos, tinctures, pastes, ointments, salves, powders, liquid or semiliquid formulation and the like. Application of said compositions may be by aerosol e.g. with a propellant such as nitrogen carbon dioxide, a freon, or without a propellant such as a pump spray, drops, lotions, or a semisolid such as a thickened composition which can be applied by a swab. In particular compositions, semisolid compositions such as salves, creams, pastes, gellies, ointments and the like will conveniently be used.

It is especially advantageous to formulate the subject compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discrete units suitable as unitary dosages, each unit containing a predetermined quantity of active ingredient calculated to
5 produce the desired therapeutic effect in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powders packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The pharmaceutical preparations of the present invention can be used, as stated
10 above, for the many applications which can be considered cosmetic uses. Cosmetic compositions known in the art, preferably hypoallergic and pH controlled are especially preferred, and include toilet waters, packs, lotions, skin milks or milky lotions. The preparations contain, besides the hedgehog or ptc therapeutic, components usually employed in such preparations. Examples of such components are oils, fats, waxes,
15 surfactants, humectants, thickening agents, antioxidants, viscosity stabilizers, chelating agents, buffers, preservatives, perfumes, dyestuffs, lower alkanols, and the like. If desired, further ingredients may be incorporated in the compositions, e.g. antiinflammatory agents, antibacterials, antifungals, disinfectants, vitamins, sunscreens, antibiotics, or other anti-acne agents.

Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate. As examples of surfactants there may be cited
25 anionic surfactants such as sodium stearate, sodium cetylsulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyltrimethylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrochloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty
30 acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g. the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin. Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of lower
35 alcohols include ethanol and isopropanol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin;

examples of chelating agents include disodium edetate and ethanehydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

5 For preparing ointments, creams, toilet waters, skin milks, and the like, typically from 0.01 to 10% in particular from 0.1 to 5% and more in particular from 0.2 to 2.5% of the active ingredient, e.g., of the hedgehog or ptc therapeutic, will be incorporated in the compositions. In ointments or creams, the carrier for example consists of 1 to 20%, in particular 5 to 15% of a humectant, 0.1 to 10% in particular from 0.5 to 5% of a thickener
10 and water; or said carrier may consist of 70 to 99%, in particular 20 to 95% of a surfactant, and 0 to 20%, in particular 2.5 to 15% of a fat; or 80 to 99.9% in particular 90 to 99% of a thickener; or 5 to 15% of a surfactant, 2-15% of a humectant, 0 to 80% of an oil, very small (< 2%) amounts of preservative, coloring agent and/or perfume, and water. In a toilet
15 water, the carrier for example consists of 2 to 10% of a lower alcohol, 0.1 to 10% or in particular 0.5 to 1% of a surfactant, 1 to 20%, in particular 3 to 7% of a humectant, 0 to 5% of a buffer, water and small amounts (< 2%) of preservative, dyestuff and/or perfume. In a skin milk, the carrier typically consists of 10-50% of oil, 1 to 10% of surfactant, 50-80% of water and 0 to 3% of preservative and/or perfume. In the aforementioned preparations, all % symbols refer to weight by weight percentage.

20 Particular compositions for use in the method of the present invention are those wherein the hedgehog or ptc therapeutic is formulated in liposome-containing compositions. Liposomes are artificial vesicles formed by amphiphatic molecules such as polar lipids, for example, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebiosides.
25 Liposomes are formed when suitable amphiphathic molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material (also referred to as coarse liposomes). Another type of liposome known to be consisting of a single bilayer encapsulating aqueous material is referred to as a unilamellar vesicle. If water-soluble
30 materials are included in the aqueous phase during the swelling of the lipids they become entrapped in the aqueous layer between the lipid bilayers.

Water-soluble active ingredients such as, for example, various salt forms of a hedgehog polypeptide, are encapsulated in the aqueous spaces between the molecular layers. The lipid soluble active ingredient of hedgehog or ptc therapeutic, such as an organic
35 mimetic, is predominantly incorporated into the lipid layers, although polar head groups may protude from the layer into the aqueous space. The encapsulation of these compounds can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation from an organic

solvent. When this film is dispersed in a suitable aqueous medium, multilamellar liposomes are formed. Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble active ingredients are usually incorporated by dispersing the cast film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysis or other art-known suitable procedures. The lipid-soluble active ingredient is usually incorporated by dissolving it in the organic solvent with the phospholipid prior to casting the film. If the solubility of the material in the lipid phase is not exceeded or the amount present is not in excess of that which can be bound to the lipid, liposomes prepared by the above method usually contain most of the material bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required.

A particularly convenient method for preparing liposome formulated forms of hedgehog and ptc therapeutics is the method described in EP-A-253,619, incorporated herein by reference. In this method, single bilayered liposomes containing encapsulated active ingredients are prepared by dissolving the lipid component in an organic medium, injecting the organic solution of the lipid component under pressure into an aqueous component while simultaneously mixing the organic and aqueous components with a high speed homogenizer or mixing means, whereupon the liposomes are formed spontaneously.

The single bilayered liposomes containing the encapsulated hedgehog or ptc therapeutic can be employed directly or they can be employed in a suitable pharmaceutically acceptable carrier for topical administration. The viscosity of the liposomes can be increased by the addition of one or more suitable thickening agents such as, for example xanthan gum, hydroxypropyl cellulose, hydroxypropyl methylcellulose and mixtures thereof. The aqueous component may consist of water alone or it may contain electrolytes, buffered systems and other ingredients, such as, for example, preservatives. Suitable electrolytes which can be employed include metal salts such as alkali metal and alkaline earth metal salts. The preferred metal salts are calcium chloride, sodium chloride and potassium chloride. The concentration of the electrolyte may vary from zero to 260 mM, preferably from 5 mM to 160 mM. The aqueous component is placed in a suitable vessel which can be adapted to effect homogenization by effecting great turbulence during the injection of the organic component. Homogenization of the two components can be accomplished within the vessel, or, alternatively, the aqueous and organic components may be injected separately into a mixing means which is located outside the vessel. In the latter case, the liposomes are formed in the mixing means and then transferred to another vessel for collection purpose.

The organic component consists of a suitable non-toxic, pharmaceutically acceptable solvent such as, for example ethanol, glycerol, propylene glycol and polyethylene glycol, and a suitable phospholipid which is soluble in the solvent. Suitable phospholipids which can be employed include lecithin, phosphatidylcholine, 5 phosphatidylserine, phosphatidylethanol-amine, phosphatidylinositol, lysophosphatidylcholine and phosphatidyl glycerol, for example. Other lipophilic additives may be employed in order to selectively modify the characteristics of the liposomes. Examples of such other additives include stearylamine, phosphatidic acid, tocopherol, cholesterol and lanolin extracts.

10 In addition, other ingredients which can prevent oxidation of the phospholipids may be added to the organic component. Examples of such other ingredients include tocopherol, butylated hydroxyanisole, butylated hydroxytoluene, ascorbyl palmitate and ascorbyl oleate. Preservatives such as benzoic acid, methyl paraben and propyl paraben may also be added.

15 Apart from the above-described compositions, use may be made of covers, e.g. plasters, bandages, dressings, gauze pads and the like, containing an appropriate amount of a hedgehog or ptc therapeutic. In some cases use may be made of plasters, bandages, dressings, gauze pads and the like which have been impregnated with a topical formulation containing the therapeutic formulation.

20 VII. Exemplary Hedgehog Antagonists

Exemplary *hedgehog* antagonists of the invention include cyclopamine, tomatidine, jervine, AY9944, triparanol, forskolin, cAMP, dibutyryl cAMP (and other hydrophobically modified cAMP variants) and functionally effective derivatives thereof. Additional *hedgehog* antagonists, functionally effective derivatives, compositions and methods for 25 making such compounds are described in detail in the following U.S. Patent applications: Baxter et al., 09/663,835; Beachy et al. entitled "Inhibitors of hedgehog signaling pathways, compositions and uses related thereto" filed October 10, 2000; Baxter et al. "Mediators of hedgehog signaling pathway, compositions and uses related thereto" filed October 13, 2000; Philip Beachy, "Regulators of the hedgehog pathway, compositions and uses related 30 thereto" filed October 13, 2000.

Evaluation of the effects of hedgehog on T-cell development

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

5 Utilizing a fetal thymic organ culture, the effects of *Shh* on T-cell maturation was determined. Briefly, immature thymocytes (CD4-/CD8- [DN]) were isolated from E14 thymus. The cells were culture for about 7 days, and the level of mature thymocytes, e.g., CD4+/CD8+ [DP], was assessed. In certain cultures, octyl-modified Shh was added to the culture; in other cultures, the anti-hedgehog 5E1 antibody was added. As illustrated in
10 Figures 1-4, the addition of Shh to the culture resulted in an increase in the percentage of cells after 7 days with markers indicative of immature thymocytes. Conversely, addition of the 5E1 antibody resulted in an increase in the percentage of mature T lymphocytes after 7 days.

15 All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

20 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.

SEQUENCE LISTING

5 (2) INFORMATION FOR SEQ ID NO:1:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1277 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 10 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: cDNA
 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1275
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

20	ATG GTC GAA ATG CTG CTG TTG ACA AGA ATT CTC TTG GTG GGC TTC ATC	48
	Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile	
	1 5 10 15	
25	TGC GCT CTT TTA GTC TCC TCT GGG CTG ACT TGT GGA CCA GGC AGG GGC	96
	Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly	
	20 25 30	
30	ATT GGA AAA AGG AGG CAC CCC AAA AAG CTG ACC CCG TTA GCC TAT AAG	144
	Ile Gly Lys Arg Arg His Pro Lys Leu Thr Pro Leu Ala Tyr Lys	
	35 40 45	
35	CAG TTT ATT CCC AAT GTG GCA GAG AAG ACC CTA GGG GCC AGT GGA AGA	192
	Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg	
	50 55 60	
40	TAT GAA GGG AAG ATC ACA AGA AAC TCC GAG AGA TTT AAA GAA CTA ACC	240
	Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr	
	65 70 75 80	
45	CCA AAT TAC AAC CCT GAC ATT ATT TTT AAG GAT GAA GAG AAC ACG GGA	288
	Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly	
	85 90 95	
50	GCT GAC AGA CTG ATG ACT CAG CGC TGC AAG GAC AAG CTG AAT GCC CTG	336
	Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu	
	100 105 110	
55	GCG ATC TCG GTG ATG AAC CAG TGG CCC GGG GTG AAG CTG CGG GTG ACC	384
	Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr	
	115 120 125	
60	GAG GGC TGG GAC GAG GAT GGC CAT CAC TCC GAG GAA TCG CTG CAC TAC	432
	Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr	
	130 135 140	
60	GAG GGT CGC GCC GTG GAC ATC ACC ACG TCG GAT CGG GAC CGC AGC AAG	480
	Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys	
	145 150 155 160	

	TAC GGA ATG CTG GCC CGC CTC GCC GTC GAG GCC GGC TTC GAC TGG GTC	528
	Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val	
	165 170 175	
5	TAC TAC GAG TCC AAG GCG CAC ATC CAC TGC TCC GTC AAA GCA GAA AAC	576
	Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn	
	180 185 190	
10	TCA GTG GCA GCG AAA TCA GGA GGC TGC TTC CCT GGC TCA GCC ACA GTG	624
	Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val	
	195 200 205	
15	CAC CTG GAG CAT GGA GGC ACC AAG CTG GTG AAG GAC CTG AGC CCT GGG	672
	His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly	
	210 215 220	
20	GAC CGC GTG CTG GCT GCT GAC GCG GAC GGC CGG CTG CTC TAC AGT GAC	720
	Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp	
	225 230 235 240	
25	TTC CTC ACC TTC CTC GAC CGG ATG GAC AGC TCC CGA AAG CTC TTC TAC	768
	Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr	
	245 250 255	
30	GTC ATC GAG ACG CGG CAG CCC CGG GCC CGG CTG CTA CTG ACG GCG GCC	816
	Val Ile Glu Thr Arg Gln Pro Arg Ala Arg Leu Leu Thr Ala Ala	
	260 265 270	
35	CAC CTG CTC TTT GTG GCC CCC CAG CAC AAC CAG TCG GAG GCC ACA GGG	864
	His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly	
	275 280 285	
40	TCC ACC AGT GGC CAG GCG CTC TTC GCC AGC AAC GTG AAG CCT GGC CAA	912
	Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln	
	290 295 300	
45	CGT GTC TAT GTG CTG GGC GAG GGC GGG CAG CAG CTG CTG CCG GCG TCT	960
	Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser	
	305 310 315 320	
50	GTC CAC AGC GTC TCA TTG CGG GAG GAG GCG TCC GGA GCC TAC GCC CCA	1008
	Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro	
	325 330 335	
55	CTC ACC GCC CAG GGC ACC ATC CTC ATC AAC CGG GTG TTG GCC TCC TGC	1056
	Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys	
	340 345 350	
60	TAC GCC GTC ATC GAG GAG CAC AGT TGG GCC CAT TGG GCC TTC GCA CCA	1104
	Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro	
	355 360 365	
65	TTC CGC TTG GCT CAG GGG CTG CTG GCC GCC CTC TGC CCA GAT GGG GCC	1152
	Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala	
	370 375 380	
70	ATC CCT ACT GCC GCC ACC ACC ACC ACT GGC ATC CAT TGG TAC TCA CGG	1200
	Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg	
	385 390 395 400	
75	CTC CTC TAC CGC ATC GGC AGC TGG GTG CTG GAT GGT GAC GCG CTG CAT	1248
	Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His	

	405	410	415	
	CCG CTG GGC ATG GTG GCA CCG GCC AGC TG			1277
5	Pro Leu Gly Met Val Ala Pro Ala Ser			
	420	425		

(2) INFORMATION FOR SEQ ID NO:2:

- 10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1190 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- 20 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1191

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

	ATG GCT CTG CCG GCC AGT CTG TTG CCC CTG TGC TGC TTG GCA CTC TTG	48
	Met Ala Leu Pro Ala Ser Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu	
	1 5 10 15	
30	GCA CTA TCT GCC CAG AGC TGC GGG CCG GGC CGA GGA CCG GTT GGC CGG	96
	Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg	
	20 25 30	
35	CGG CGT TAT GTG CGC AAG CAA CTT GTG CCT CTG CTA TAC AAG CAG TTT	144
	Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe	
	35 40 45	
40	GTG CCC AGT ATG CCC GAG CGG ACC CTG GGC GCG AGT GGG CCA GCG GAG	192
	Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu	
	50 55 60	
	GGG AGG GTA ACA AGG GGG TCG GAG CGC TTC CGG GAC CTC GTA CCC AAC	240
45	Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn	
	65 70 75 80	
	TAC AAC CCC GAC ATA ATC TTC AAG GAT GAG GAG AAC AGC GGC GCA GAC	288
	Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp	
	85 90 95	
50	CGC CTG ATG ACA GAG CGT TGC AAA GAG CGG GTG AAC GCT CTA GCC ATC	336
	Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile	
	100 105 110	
55	GCG GTG ATG AAC ATG TGG CCC GGA GTA CGC CTA CGT GTG ACT GAA GGC	384
	Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly	
	115 120 125	
60	TGG GAC GAG GAC GGC CAC CAC GCA CAG GAT TCA CTC CAC TAC GAA GGC	432
	Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly	
	130 135 140	

5	CGT GCC TTG GAC ATC ACC ACG TCT GAC CGT GAC CGT AAT AAG TAT GGT	480
	Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly	
	145 150 155 160	
10	TTG TTG GCG CGC CTA GCT GTG GAA GCC GGA TTC GAC TGG GTC TAC TAC	528
	Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr	
	165 170 175	
15	GAG TCC CGC AAC CAC ATC CAC GTA TCG GTC AAA GCT GAT AAC TCA CTG	576
	Glu Ser Arg Asn His Ile His Val Ser Val Lys Ala Asp Asn Ser Leu	
	180 185 190	
20	GCG GTC CGA GCC GGA GGC TGC TTT CCG GGA AAT GCC ACG GTG CGC TTG	624
	Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu	
	195 200 205	
25	CGG AGC GGC GAA CGG AAG GGG CTG AGG GAA CTA CAT CGT GGT GAC TGG	672
	Arg Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp	
	210 215 220	
30	GTA CTG GCC GCT GAT GCA GCG GGC CGA GTG GTA CCC ACG CCA GTG CTG	720
	Val Leu Ala Ala Asp Ala Ala Gly Arg Val Val Pro Thr Pro Val Leu	
	225 230 235 240	
35	CTC TTC CTG GAC CGG GAT CTG CAG CGC CGC GCC TCG TTC GTG GCT GTG	768
	Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val	
	245 250 255	
40	GAG ACC GAG CGG CCT CCG CGC AAA CTG TTG CTC ACA CCC TGG CAT CTG	816
	Glu Thr Glu Arg Pro Pro Arg Lys Leu Leu Thr Pro Trp His Leu	
	260 265 270	
45	GTG TTC GCT GCT CGC GGG CCA GCG CCT GCT CCA GGT GAC TTT GCA CCG	864
	Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro	
	275 280 285	
50	GTG TTC GCG CGC CGC TTA CGT GCT GGC GAC TCG GTG CTG GCT CCC GGC	912
	Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly	
	290 295 300	
55	GGG GAC GCG CTC CAG CCG GCG CGC GTA GCC CGC GTG GCG CGC GAG GAA	960
	Gly Asp Ala Leu Gln Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu	
	305 310 315 320	
60	GCC GTG GGC GTG TTC GCA CCG CTC ACT GCG CAC GGG ACG CTG CTG GTC	1008
	Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val	
	325 330 335	
65	AAC GAC GTC CTC GCC TCC TGC TAC GCG GTT CTA GAG AGT CAC CAG TGG	1056
	Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp	
	340 345 350	
70	GCC CAC CGC GCC TTC GCC CCT TTG CGG CTG CTG CAC GCG CTC GGG GCT	1104
	Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala	
	355 360 365	
75	CTG CTC CCT GGG GGT GCA GTC CAG CCG ACT GGC ATG CAT TGG TAC TCT	1152
	Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser	
	370 375 380	

CGC CTC CTT TAC CGC TTG GCC GAG GAG TTA ATG GGC TG 1190
 Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
 385 390 395

5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 1281 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..1233

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG TCT CCC GCC TGG CTC CGG CCC CGA CTG CGG TTC TGT CTG TTC CTG 48
 Met Ser Pro Ala Trp Leu Arg Pro Arg Leu Arg Phe Cys Leu Phe Leu
 1 5 10 15

CTG CTG CTG CTT CTG GTG CCG GCG GCG CGG GGC TGC GGG CCG GGC CGG 96
 Leu Leu Leu Leu Leu Val Pro Ala Ala Arg Gly Cys Gly Pro Gly Arg
 20 25 30

GTG GTG GGC AGC CGC CGG AGG CCG CCT CGC AAG CTC GTG CCT CTT GCC 144
 Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
 35 40 45

TAC AAG CAG TTC AGC CCC AAC GTG CCG GAG AAG ACC CTG GGC GCC AGC 192
 Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
 50 55 60

GGG CGC TAC GAA GGC AAG ATC GCG CGC AGC TCT GAG CGC TTC AAA GAG 240
 Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65 70 75 80

CTC ACC CCC AAC TAC AAT CCC GAC ATC ATC TTC AAG GAC GAG GAG AAC 288
 Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
 85 90 95

ACG GGT GCC GAC CGC CTC ATG ACC CAG CGC TGC AAG GAC CGT CTG AAC 336
 Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
 100 105 110

TCA CTG GCC ATC TCT GTC ATG AAC CAG TGG CCT GGT GTG AAA CTG CGG 384
 Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
 115 120 125

GTG ACC GAA GGC CGG GAT GAA GAT GGC CAT CAC TCA GAG GAG TCT TTA 432
 Val Thr Glu Gly Arg Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
 130 135 140

CAC TAT GAG GGC CGC GCG GTG GAT ATC ACC ACC TCA GAC CGT GAC CGA 480
 His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg

	145				150					155				160	
	AAT AAG TAT GGA CTG CTG GCG CGC TTA GCA GTG GAG GCC GGC TTC GAC														528
5	Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp				165				170					175	
	TGG GTG TAT TAC GAG TCC AAG GCC CAC GTG CAT TGC TCT GTC AAG TCT														576
	Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser			180				185					190		
10	GAG CAT TCG GCC GCT GCC AAG ACA GGT GGC TGC TTT CCT GCC GGA GCC														624
	Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala			195			200					205			
15	CAG GTG CGC CTA GAG AAC GGG GAG CGT GTG GCC CTG TCA GCT GTA AAG														672
	Gln Val Arg Leu Glu Asn Gly Glu Arg Val Ala Leu Ser Ala Val Lys			210			215					220			
	CCA GGA GAC CGG GTG CTG GCC ATG GGG GAG GAT GGG ACC CCC ACC TTC														720
20	Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Thr Pro Thr Phe				225		230			235				240	
	AGT GAT GTG CTT ATT TTC CTG GAC CGC GAG CCA AAC CGG CTG AGA GCT														768
25	Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro Asn Arg Leu Arg Ala				245				250					255	
	TTC CAG GTC ATC GAG ACT CAG GAT CCT CCG CGT CGG CTG GCG CTC ACG														816
	Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr			260				265					270		
30	CCT GCC CAC CTG CTC TTC ATT GCG GAC AAT CAT ACA GAA CCA GCA GCC														864
	Pro Ala His Leu Leu Phe Ile Ala Asp Asn His Thr Glu Pro Ala Ala			275			280					285			
35	CAC TTC CGG GCC ACA TTT GCC AGC CAT GTG CAA CCA GGC CAA TAT GTG														912
	His Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val			290			295					300			
	CTG GTA TCA GGG GTA CCA GGC CTC CAG CCT GCT CGG GTG GCA GCT GTC														960
40	Leu Val Ser Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val				305		310			315				320	
	TCC ACC CAC GTG GCC CTT GGG TCC TAT GCT CCT CTC ACA AGG CAT GGG														1008
45	Ser Thr His Val Ala Leu Gly Ser Tyr Ala Pro Leu Thr Arg His Gly				325				330					335	
	ACA CTT GTG GTG GAG GAT GTG GTG GCC TCC TGC TTT GCA GCT GTG GCT														1056
	Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala			340				345					350		
50	GAC CAC CAT CTG GCT CAG TTG GCC TTC TGG CCC CTG CGA CTG TTT CCC														1104
	Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe Pro			355			360					365			
55	AGT TTG GCA TGG GGC AGC TGG ACC CCA AGT GAG GGT GTT CAC TCC TAC														1152
	Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu Gly Val His Ser Tyr			370			375					380			
	CCT CAG ATG CTC TAC CGC CTG GGG CGT CTC TTG CTA GAA GAG AGC ACC														1200
60	Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Ser Thr				385		390			395				400	

TTC CAT CCA CTG GGC ATG TCT GGG GCA GGA AGC TGAAGGGACT CTAACCACTG 1253
 Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
 405 410

5

CCCTCCTGGA ACTGCTGTGC GTGGATCC 1281

10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1313 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

15

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1314

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATG CTG CTG CTG CTG GCC AGA TGT TTT CTG GTG ATC CTT GCT TCC TCG 48
 Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser
 1 5 10 15

30

CTG CTG GTG TGC CCC GGG CTG GCC TGT GGG CCC GGC AGG GGG TTT GGA 96
 Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly
 20 25 30

35

AAG AGG CGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT 144
 Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe
 35 40 45

40

ATT CCC AAC GTA GCC GAG AAG ACC CTA GGG GCC AGC GGC AGA TAT GAA 192
 Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu
 50 55 60

45

GGG AAG ATC ACA AGA AAC TCC GAA CGA TTT AAG GAA CTC ACC CCC AAT 240
 Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn
 65 70 75 80

50

TAC AAC CCC GAC ATC ATA TTT AAG GAT GAG GAA AAC ACG GGA GCA GAC 288
 Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp
 85 90 95

CGG CTG ATG ACT CAG AGG TGC AAA GAC AAG TTA AAT GCC TTG GCC ATC 336
 Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile
 100 105 110

55

TCT GTG ATG AAC CAG TGG CCT GGA GTG AGG CTG CGA GTG ACC GAG GGC 384
 Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
 115 120 125

60

TGG GAT GAG GAC GGC CAT CAT TCA GAG GAG TCT CTA CAC TAT GAG GGT 432
 Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly
 130 135 140

5	CGA GCA GTG GAC ATC ACC ACG TCC GAC CGG GAC CGC AGC AAG TAC GGC	480
	Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly	
	145 150 155 160	
10	ATG CTG GCT CGC CTG GCT GTG GAA GCA GGT TTC GAC TGG GTC TAC TAT	528
	Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr	
	165 170 175	
15	GAA TCC AAA GCT CAC ATC CAC TGT TCT GTG AAA GCA GAG AAC TCC GTG	576
	Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val	
	180 185 190	
20	GCG GCC AAA TCC GGC GGC TGT TTC CCG GGA TCC GCC ACC GTG CAC CTG	624
	Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu	
	195 200 205	
25	GAG CAG GGC GGC ACC AAG CTG GTG AAG GAC TTA CGT CCC GGA GAC CGC	672
	Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg	
	210 215 220	
30	GTG CTG GCG GCT GAC GAC CAG GGC CGG CTG CTG TAC AGC GAC TTC CTC	720
	Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu	
	225 230 235 240	
35	ACC TTC CTG GAC CGC GAC GAA GGC GCC AAG AAG GTC TTC TAC GTG ATC	768
	Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile	
	245 250 255	
40	GAG ACG CTG GAG CCG CGC GAG CGC CTG CTG CTC ACC GCC GCG CAC CTG	816
	Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu	
	260 265 270	
45	CTC TTC GTG GCG CCG CAC AAC GAC TCG GGG CCC ACG CCC GGG CCA AGC	864
	Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser	
	275 280 285	
50	GCG CTC TTT GCC AGC CGC GTG CGC CCC GGG CAG CGC GTG TAC GTG GTG	912
	Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val	
	290 295 300	
55	GCT GAA CGC GGC GGG GAC CGC CGG CTG CTG CCC GCC GCG GTG CAC AGC	960
	Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser	
	305 310 315 320	
60	GTG ACG CTG CGA GAG GAG GAG GCG GGC GCG TAC GCG CCG CTC ACG GCG	1008
	Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala	
	325 330 335	
65	CAC GGC ACC ATT CTC ATC AAC CGG GTG CTC GCC TCG TGC TAC GCT GTC	1056
	His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val	
	340 345 350	
70	ATC GAG GAG CAC AGC TGG GCA CAC CGG GCC TTC GCG CCT TTC CGC CTG	1104
	Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu	
	355 360 365	
75	GCG CAC GCG CTG CTG GCC GCG CTG GCA CCC GCC CGC ACG GAC GGC GGC	1152
	Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly	
	370 375 380	

	GGC GGC GGC AGC ATC CCT GCA GCG CAA TCT GCA ACG GAA GCG AGG GGC	1200
	Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly	
	385 390 395 400	
5	GCG GAG CCG ACT GCG GGC ATC CAC TGG TAC TCG CAG CTG CTC TAC CAC	1248
	Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His	
	405 410 415	
10	ATT GGC ACC TGG CTG TTG GAC AGC GAG ACC ATG CAT CCC TTG GGA ATG	1296
	Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met	
	420 425 430	
15	GCG GTC AAG TCC AGC TG	1313
	Ala Val Lys Ser Ser	
	435	
(2) INFORMATION FOR SEQ ID NO:5:		
20	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 1256 base pairs	
	(B) TYPE: nucleic acid	
	(C) STRANDEDNESS: both	
	(D) TOPOLOGY: linear	
25	(ii) MOLECULE TYPE: cDNA	
30	(ix) FEATURE:	
	(A) NAME/KEY: CDS	
	(B) LOCATION: 1..1257	
35	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:	
	ATG CGG CTT TTG ACG AGA GTG CTG CTG GTG TCT CTT CTC ACT CTG TCC	48
	Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser	
	1 5 10 15	
40	TTG GTG GTG TCC GGA CTG GCC TGC GGT CCT GGC AGA GGC TAC GGC AGA	96
	Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg	
	20 25 30	
45	AGA AGA CAT CCG AAG AAG CTG ACA CCT CTC GCC TAC AAG CAG TTC ATA	144
	Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile	
	35 40 45	
50	CCT AAT GTC GCG GAG AAG ACC TTA GGG GCC AGC GGC AGA TAC GAG GGC	192
	Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly	
	50 55 60	
55	AAG ATA ACG CGC AAT TCG GAG AGA TTT AAA GAA CTT ACT CCA AAT TAC	240
	Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr	
	65 70 75 80	
60	AAT CCC GAC ATT ATC TTT AAG GAT GAG GAG AAC ACG GGA GCG GAC AGG	288
	Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg	
	85 90 95	
60	CTC ATG ACA CAG AGA TGC AAA GAC AAG CTG AAC TCG CTG GCC ATC TCT	336
	Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ser Leu Ala Ile Ser	

	100	105	110	
5	GTA ATG AAC CAC TGG CCA GGG GTT AAG CTG CGT GTG ACA GAG GGC TGG Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp 115 120 125	384		
10	GAT GAG GAC GGT CAC CAT TTT GAA GAA TCA CTC CAC TAC GAG GGA AGA Asp Glu Asp Gly His His Phe Glu Glu Ser Leu His Tyr Glu Gly Arg 130 135 140	432		
15	GCT GTT GAT ATT ACC ACC TCT GAC CGA GAC AAG AGC AAA TAC GGG ACA Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys Tyr Gly Thr 145 150 155 160	480		
20	CTG TCT CGC CTA GCT GTG GAG GCT GGA TTT GAC TGG GTC TAT TAC GAG Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu 165 170 175	528		
25	TCC AAA GCC CAC ATT CAT TGC TCT GTC AAA GCA GAA AAT TCG GTT GCT Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala 180 185 190	576		
30	GCG AAA TCT GGG GGC TGT TTC CCA GGT TCG GCT CTG GTC TCG CTC CAG Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Leu Val Ser Leu Gln 195 200 205	624		
35	GAC GGA GGA CAG AAG GCC GTG AAG GAC CTG AAC CCC GGA GAC AAG GTG Asp Gly Gly Gln Lys Ala Val Lys Asp Leu Asn Pro Gly Asp Lys Val 210 215 220	672		
40	CTG GCG GCA GAC AGC GCG GGA AAC CTG GTG TTC AGC GAC TTC ATC ATG Leu Ala Ala Asp Ser Ala Gly Asn Leu Val Phe Ser Asp Phe Ile Met 225 230 235 240	720		
45	TTC ACA GAC CGA GAC TCC ACG ACG CGA CGT GTG TTT TAC GTC ATA GAA Phe Thr Asp Arg Asp Ser Thr Thr Arg Arg Val Phe Tyr Val Ile Glu 245 250 255	768		
50	ACG CAA GAA CCC GTT GAA AAG ATC ACC CTC ACC GCC GCT CAC CTC CTT Thr Gln Glu Pro Val Glu Lys Ile Thr Leu Thr Ala Ala His Leu Leu 260 265 270	816		
55	TTT GTC CTC GAC AAC TCA ACG GAA GAT CTC CAC ACC ATG ACC GCC GCG Phe Val Leu Asp Asn Ser Thr Glu Asp Leu His Thr Met Thr Ala Ala 275 280 285	864		
60	TAT GCC AGC AGT GTC AGA GCC GGA CAA AAG GTG ATG GTT GTT GAT GAT Tyr Ala Ser Ser Val Arg Ala Gly Gln Lys Val Met Val Val Asp Asp 290 295 300	912		
65	AGC GGT CAG CTT AAA TCT GTC ATC GTG CAG CGG ATA TAC ACG GAG GAG Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu 305 310 315 320	960		
70	CAG CGG GGC TCG TTC GCA CCA GTG ACT GCA CAT GGG ACC ATT GTG GTC Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val 325 330 335	1008		
75	GAC AGA ATA CTG GCG TCC TGT TAC GCC GTA ATA GAG GAC CAG GGG CTT Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu 340 345 350	1056		

5 GCG CAT TTG GCC TTC GCG CCC GCC AGG CTC TAT TAT TAC GTG TCA TCA 1104
 Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
 355 360 365
 TTC CTG TCC CCC AAA ACT CCA GCA GTC GGT CCA ATG CGA CTT TAC AAC 1152
 Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
 370 375 380
 10 AGG AGG GGG TCC ACT GGT ACT CCA GGC TCC TGT CAT CAA ATG GGA ACG 1200
 Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
 385 390 395 400
 15 TGG CTT TTG GAC AGC AAC ATG CTT CAT CCT TTG GGG ATG TCA GTA AAC 1248
 Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
 405 410 415
 TCA AGC TG 1256
 Ser Ser
 20

(2) INFORMATION FOR SEQ ID NO:6:

25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1425 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

35 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1425

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

48 ATG CTG CTG CTG GCG AGA TGT CTG CTG CTA GTC CTC GTC TCC TCG CTG
 Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
 1 5 10 15
 96 CTG GTA TGC TCG GGA CTG GCG TGC GGA CCG GGC AGG GGG TTC GGG AAG
 Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
 20 25 30
 144 AGG AGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT ATC
 Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
 35 40 45
 192 CCC AAT GTG GCC GAG AAG ACC CTA GGC GCC AGC GGA AGG TAT GAA GGG
 Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
 50 55 60
 240 AAG ATC TCC AGA AAC TCC GAG CGA TTT AAG GAA CTC ACC CCC AAT TAC
 Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
 65 70 75 80
 288 AAC CCC GAC ATC ATA TTT AAG GAT GAA GAA AAC ACC GGA GCG GAC AGG

	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	Arg	
				85						90					95		
5	CTG	ATG	ACT	CAG	AGG	TGT	AAG	GAC	AAG	TTG	AAC	GCT	TTG	GCC	ATC	TCG	336
	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ala	Leu	Ala	Ile	Ser	
				100					105					110			
10	GTG	ATG	AAC	CAG	TGG	CCA	GGA	GTG	AAA	CTG	CGG	GTG	ACC	GAG	GGC	TGG	384
	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	
				115					120					125			
15	GAC	GAA	GAT	GGC	CAC	CAC	TCA	GAG	GAG	TCT	CTG	CAC	TAC	GAG	GGC	CGC	432
	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	
				130					135					140			
20	GCA	GTG	GAC	ATC	ACC	ACG	TCT	GAC	CGC	GAC	CGC	AGC	AAG	TAC	GGC	ATG	480
	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Ser	Lys	Tyr	Gly	Met	
							150						155			160	
25	CTG	GCC	CGC	CTG	GCG	GTG	GAG	GCC	GGC	TTC	GAC	TGG	GTG	TAC	TAC	GAG	528
	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	
							165				170					175	
30	TCC	AAG	GCA	CAT	ATC	CAC	TGC	TCG	GTG	AAA	GCA	GAG	AAC	TCG	GTG	GCG	576
	Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala	
				180						185				190			
35	GCC	AAA	TCG	GGA	GGC	TGC	TTC	CCG	GGC	TCG	GCC	ACG	GTG	CAC	CTG	GAG	624
	Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Thr	Val	His	Leu	Glu	
				195					200					205			
40	CAG	GGC	GGC	ACC	AAG	CTG	GTG	AAG	GAC	CTG	AGC	CCC	GGG	GAC	CGC	GTG	672
	Gln	Gly	Gly	Thr	Lys	Leu	Val	Lys	Asp	Leu	Ser	Pro	Gly	Asp	Arg	Val	
				210					215				220				
45	CTG	GCG	GCG	GAC	GAC	CAG	GGC	CGG	CTG	CTC	TAC	AGC	GAC	TTC	CTC	ACT	720
	Leu	Ala	Ala	Asp	Asp	Gln	Gly	Arg	Leu	Leu	Tyr	Ser	Asp	Phe	Leu	Thr	
							230					235				240	
50	TTC	CTG	GAC	CGC	GAC	GAC	GGC	GCC	AAG	AAG	GTC	TTC	TAC	GTG	ATC	GAG	768
	Phe	Leu	Asp	Arg	Asp	Asp	Gly	Ala	Lys	Lys	Val	Phe	Tyr	Val	Ile	Glu	
							245				250					255	
55	ACG	CGG	GAG	CCG	CGC	GAG	CGC	CTG	CTG	CTC	ACC	GCC	GCG	CAC	CTG	CTC	816
	Thr	Arg	Glu	Pro	Arg	Glu	Arg	Leu	Leu	Leu	Thr	Ala	Ala	His	Leu	Leu	
				260						265					270		
60	TTT	GTG	GCG	CCG	CAC	AAC	GAC	TCG	GCC	ACC	GGG	GAG	CCC	GAG	GCG	TCC	864
	Phe	Val	Ala	Pro	His	Asn	Asp	Ser	Ala	Thr	Gly	Glu	Pro	Glu	Ala	Ser	
				275					280					285			
65	TCG	GGC	TCG	GGG	CCG	CCT	TCC	GGG	GGC	GCA	CTG	GGG	CCT	CGG	GCG	CTG	912
	Ser	Gly	Ser	Gly	Pro	Pro	Ser	Gly	Gly	Ala	Leu	Gly	Pro	Arg	Ala	Leu	
				290					295				300				
70	TTC	GCC	AGC	CGC	GTG	CGC	CCG	GGC	CAG	CGC	GTG	TAC	GTG	GTG	GCC	GAG	960
	Phe	Ala	Ser	Arg	Val	Arg	Pro	Gly	Gln	Arg	Val	Tyr	Val	Val	Ala	Glu	
							310					315				320	
75	CGT	GAC	GGG	GAC	CGC	CGG	CTC	CTG	CCC	GCC	GCT	GTG	CAC	AGC	GTG	ACC	1008
	Arg	Asp	Gly	Asp	Arg	Arg	Leu	Leu	Pro	Ala	Ala	Val	His	Ser	Val	Thr	

		325		330		335		
5	CTA AGC GAG GAG GCC GCG GGC GCC TAC GCG CCG CTC ACG GCC CAG GGC	1056						
	Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly							
	340		345		350			
10	ACC ATT CTC ATC AAC CGG GTG CTG GCC TCG TGC TAC GCG GTC ATC GAG	1104						
	Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu							
	355		360		365			
15	GAG CAC AGC TGG GCG CAC CGG GCC TTC GCG CCC TTC CGC CTG GCG CAC	1152						
	Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His							
	370		375		380			
20	GCG CTC CTG GCT GCA CTG GCG CCC GCG CGC ACG GAC CGC GGC GGG GAC	1200						
	Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp							
	385		390		395		400	
25	AGC GGC GGC GGG GAC CGC GGG GGC GGC GGC GGC AGA GTA GCC CTA ACC	1248						
	Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr							
	405		410		415			
30	GCT CCA GGT GCT GCC GAC GCT CCG GGT GCG GGG GCC ACC GCG GGC ATC	1296						
	Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile							
	420		425		430			
35	CAC TGG TAC TCG CAG CTG CTC TAC CAA ATA GGC ACC TGG CTC CTG GAC	1344						
	His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp							
	435		440		445			
40	AGC GAG GCC CTG CAC CCG CTG GGC ATG GCG GTC AAG TCC AGC NNN AGC	1392						
	Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser							
	450		455		460			
45	CGG GGG GCC GGG GGA GGG GCG CGG GAG GGG GCC	1425						
	Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala							
	465		470		475			
50	(2) INFORMATION FOR SEQ ID NO:7:							
55	(i) SEQUENCE CHARACTERISTICS:							
	(A) LENGTH: 1622 base pairs							
	(B) TYPE: nucleic acid							
	(C) STRANDEDNESS: both							
	(D) TOPOLOGY: linear							
60	(ii) MOLECULE TYPE: cDNA							
	(ix) FEATURE:							
	(A) NAME/KEY: CDS							
	(B) LOCATION: 51..1283							
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:							
65	CATCAGCCCA CCAGGAGACC TCGCCCGCCG CTCCCCGGG CTCCCCGGCC ATG TCT	56						
	Met Ser							
	1							

5	CCC	GCC	CGG	CTC	CGG	CCC	CGA	CTG	CAC	TTC	TGC	CTG	GTC	CTG	TTG	CTG	104
	Pro	Ala	Arg	Leu	Arg	Pro	Arg	Leu	His	Phe	Cys	Leu	Val	Leu	Leu	Leu	
			5					10					15				
	CTG	CTG	GTG	GTG	CCC	GCG	GCA	TGG	GGC	TGC	GGG	CCG	GGT	CGG	GTG	GTG	152
	Leu	Leu	Val	Val	Pro	Ala	Ala	Trp	Gly	Cys	Gly	Pro	Gly	Arg	Val	Val	
		20					25					30					
10	GGC	AGC	CGC	CGG	CGA	CCG	CCA	CGC	AAA	CTC	GTG	CCG	CTC	GCC	TAC	AAG	200
	Gly	Ser	Arg	Arg	Arg	Pro	Pro	Arg	Lys	Leu	Val	Pro	Leu	Ala	Tyr	Lys	
		35					40				45					50	
	CAG	TTC	AGC	CCC	AAT	GTG	CCC	GAG	AAG	ACC	CTG	GGC	GCC	AGC	GGA	CGC	248
	Gln	Phe	Ser	Pro	Asn	Val	Pro	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	
					55					60					65		
20	TAT	GAA	GGC	AAG	ATC	GCT	CGC	AGC	TCC	GAG	CGC	TTC	AAG	GAG	CTC	ACC	296
	Tyr	Glu	Gly	Lys	Ile	Ala	Arg	Ser	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	
				70					75					80			
	CCC	AAT	TAC	AAT	CCA	GAC	ATC	ATC	TTC	AAG	GAC	GAG	GAG	AAC	ACA	GGC	344
	Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	
			85					90					95				
25	GCC	GAC	CGC	CTC	ATG	ACC	CAG	CGC	TGC	AAG	GAC	CGC	CTG	AAC	TCG	CTG	392
	Ala	Asp	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Arg	Leu	Asn	Ser	Leu	
		100					105					110					
	GCT	ATC	TCG	GTG	ATG	AAC	CAG	TGG	CCC	GGT	GTG	AAG	CTG	CGG	GTG	ACC	440
	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	
	115					120				125					130		
35	GAG	GGC	TGG	GAC	GAG	GAC	GGC	CAC	CAC	TCA	GAG	GAG	TCC	CTG	CAT	TAT	488
	Glu	Gly	Trp	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	His	Tyr	
					135					140					145		
	GAG	GGC	CGC	GCG	GTG	GAC	ATC	ACC	ACA	TCA	GAC	CGC	GAC	CGC	AAT	AAG	536
	Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	Asn	Lys	
				150					155					160			
45	TAT	GGA	CTG	CTG	GCG	CGC	TTG	GCA	GTG	GAG	GCC	GGC	TTT	GAC	TGG	GTG	584
	Tyr	Gly	Leu	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	
			165					170					175				
	TAT	TAC	GAG	TCA	AAG	GCC	CAC	GTG	CAT	TGC	TCC	GTC	AAG	TCC	GAG	CAC	632
	Tyr	Tyr	Glu	Ser	Lys	Ala	His	Val	His	Cys	Ser	Val	Lys	Ser	Glu	His	
		180					185					190					
50	TCG	GCC	GCA	GCC	AAG	ACG	GGC	GGC	TGC	TTC	CCT	GCC	GGA	GCC	CAG	GTA	680
	Ser	Ala	Ala	Ala	Lys	Thr	Gly	Gly	Cys	Phe	Pro	Ala	Gly	Ala	Gln	Val	
		195				200					205				210		
	CGC	CTG	GAG	AGT	GGG	GCG	CGT	GTG	GCC	TTG	TCA	GCC	GTG	AGG	CCG	GGA	728
	Arg	Leu	Glu	Ser	Gly	Ala	Arg	Val	Ala	Leu	Ser	Ala	Val	Arg	Pro	Gly	
					215					220					225		
60	GAC	CGT	GTG	CTG	GCC	ATG	GGG	GAG	GAT	GGG	AGC	CCC	ACC	TTC	AGC	GAT	776
	Asp	Arg	Val	Leu	Ala	Met	Gly	Glu	Asp	Gly	Ser	Pro	Thr	Phe	Ser	Asp	
					230				235					240			

	GTG CTC ATT TTC CTG GAC CGC GAG CCC CAC AGG CTG AGA GCC TTC CAG	824
	Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala Phe Gln	
	245 250 255	
5	GTC ATC GAG ACT CAG GAC CCC CCA CGC CGC CTG GCA CTC ACA CCC GCT	872
	Val Ile Glu Thr Gln Asp Pro Arg Arg Leu Ala Leu Thr Pro Ala	
	260 265 270	
10	CAC CTG CTC TTT ACG GCT GAC AAT CAC ACG GAG CCG GCA GCC CGC TTC	920
	His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala Arg Phe	
	275 280 285 290	
15	CGG GCC ACA TTT GCC AGC CAC GTG CAG CCT GGC CAG TAC GTG CTG GTG	968
	Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val Leu Val	
	295 300 305	
20	GCT GGG GTG CCA GGC CTG CAG CCT GCC CGC GTG GCA GCT GTC TCT ACA	1016
	Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val Ser Thr	
	310 315 320	
25	CAC GTG GCC CTC GGG GCC TAC GCC CCG CTC ACA AAG CAT GGG ACA CTG	1064
	His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly Thr Leu	
	325 330 335	
30	GTG GTG GAG GAT GTG GTG GCA TCC TGC TTC GCG GCC GTG GCT GAC CAC	1112
	Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Val Ala Asp His	
	340 345 350	
35	CAC CTG GCT CAG TTG GCC TTC TGG CCC CTG AGA CTC TTT CAC AGC TTG	1160
	His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His Ser Leu	
	355 360 365 370	
40	GCA TGG GGC AGC TGG ACC CCG GGG GAG GGT GTG CAT TGG TAC CCC CAG	1208
	Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr Pro Gln	
	375 380 385	
45	CTG CTC TAC CGC CTG GGG CGT CTC CTG CTA GAA GAG GGC AGC TTC CAC	1256
	Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser Phe His	
	390 395 400	
50	CCA CTG GGC ATG TCC GGG GCA GGG AGC TGAAAGGACT CCACCGCTGC	1303
	Pro Leu Gly Met Ser Gly Ala Gly Ser	
	405 410	
55	CCTCCTGGAA CTGCTGTACT GGGTCCAGAA GCCTCTCAGC CAGGAGGGAG CTGGCCCTGG	1363
	AAGGGACCTG AGCTGGGGGA CACTGGCTCC TGCCATCTCC TCTGCCATGA AGATACACCA	1423
	TTGAGACTTG ACTGGGCAAC ACCAGCGTCC CCCACCCGCG TCGTGGTGTA GTCATAGAGC	1483
	TGCAAGCTGA GCTGGCGAGG GGATGGTTGT TGACCCCTCT CTCCTAGAGA CCTTGAGGCT	1543
	GGCACGGCGA CTCCCAACTC AGCCTGCTCT CACTACGAGT TTTCATACTC TGCCTCCCCC	1603
	ATTGGGAGGG CCCATTCCC	1622

(2) INFORMATION FOR SEQ ID NO:8:

- 60 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1191 base pairs
 (B) TYPE: nucleic acid

(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..1191

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

15	ATG GCT CTC CTG ACC AAT CTA CTG CCC TTG TGC TGC TTG GCA CTT CTG	48
	Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu	
	1 5 10 15	
20	GCG CTG CCA GCC CAG AGC TGC GGG CCG GGC CGG GGG CCG GTT GGC CGG	96
	Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg	
	20 25 30	
25	CGC CGC TAT GCG CGC AAG CAG CTC GTG CCG CTA CTC TAC AAG CAA TTT	144
	Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe	
	35 40 45	
30	GTG CCC GGC GTG CCA GAG CGG ACC CTG GGC GCC AGT GGG CCA GCG GAG	192
	Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu	
	50 55 60	
35	GGG AGG GTG GCA AGG GGC TCC GAG CGC TTC CGG GAC CTC GTG CCC AAC	240
	Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn	
	65 70 75 80	
40	TAC AAC CCC GAC ATC ATC TTC AAG GAT GAG GAG AAC AGT GGA GCC GAC	288
	Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp	
	85 90 95	
45	CGC CTG ATG ACC GAG CGT TGC AAG GAG AGG GTG AAC GCT TTG GCC ATT	336
	Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile	
	100 105 110	
50	GCC GTG ATG AAC ATG TGG CCC GGA GTG CGC CTA CGA GTG ACT GAG GGC	384
	Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly	
	115 120 125	
55	TGG GAC GAG GAC GGC CAC CAC GCT CAG GAT TCA CTC CAC TAC GAA GGC	432
	Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly	
	130 135 140	
60	CGT GCT TTG GAC ATC ACT ACG TCT GAC CGC GAC CGC AAC AAG TAT GGG	480
	Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly	
	145 150 155 160	
65	TTG CTG GCG CGC CTC GCA GTG GAA GCC GGC TTC GAC TGG GTC TAC TAC	528
	Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr	
	165 170 175	
70	GAG TCC CGC AAC CAC GTC CAC GTG TCG GTC AAA GCT GAT AAC TCA CTG	576
	Glu Ser Arg Asn His Val His Val Ser Val Lys Ala Asp Asn Ser Leu	
	180 185 190	

	GCG GTC CGG GCG GGC GGC TGC TTT CCG GGA AAT GCA ACT GTG CGC CTG	624
	Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu	
	195 200 205	
5	TGG AGC GGC GAG CGG AAA GGG CTG CGG GAA CTG CAC CGC GGA GAC TGG	672
	Trp Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp	
	210 215 220	
10	GTT TTG GCG GCC GAT GCG TCA GGC CGG GTG GTG CCC ACG CCG GTG CTG	720
	Val Leu Ala Ala Asp Ala Ser Gly Arg Val Val Pro Thr Pro Val Leu	
	225 230 235 240	
15	CTC TTC CTG GAC CGG GAC TTG CAG CGC CGG GCT TCA TTT GTG GCT GTG	768
	Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val	
	245 250 255	
20	GAG ACC GAG TGG CCT CCA CGC AAA CTG TTG CTC ACG CCC TGG CAC CTG	816
	Glu Thr Glu Trp Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu	
	260 265 270	
25	GTG TTT GCC GCT CGA GGG CCG GCG CCC GCG CCA GGC GAC TTT GCA CCG	864
	Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro	
	275 280 285	
30	GTG TTC GCG CGC CGG CTA CGC GCT GGG GAC TCG GTG CTG GCG CCC GGC	912
	Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly	
	290 295 300	
35	GGG GAT GCG CTT CGG CCA GCG CGC GTG GCC CGT GTG GCG CGG GAG GAA	960
	Gly Asp Ala Leu Arg Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu	
	305 310 315 320	
40	GCC GTG GGC GTG TTC GCG CCG CTC ACC GCG CAC GGG ACG CTG CTG GTG	1008
	Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val	
	325 330 335	
45	AAC GAT GTC CTG GCC TCT TGC TAC GCG GTT CTG GAG AGT CAC CAG TGG	1056
	Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp	
	340 345 350	
50	GCG CAC CGC GCT TTT GCC CCC TTG AGA CTG CTG CAC GCG CTA GGG GCG	1104
	Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala	
	355 360 365	
55	CTG CTC CCC GGC GGG GCC GTC CAG CCG ACT GGC ATG CAT TGG TAC TCT	1152
	Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser	
	370 375 380	
60	CGG CTC CTC TAC CGC TTA GCG GAG GAG CTA CTG GGC TG	1191
	Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Leu Gly	
	385 390 395	

55 (2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1251 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1248

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATG GAC GTA AGG CTG CAT CTG AAG CAA TTT GCT TTA CTG TGT TTT ATC	48
Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile	
1 5 10 15	
15 AGC TTG CTT CTG ACG CCT TGT GGA TTA GCC TGT GGT CCT GGT AGA GGT	96
Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly	
20 25 30	
20 TAT GGA AAA CGA AGA CAC CCA AAG AAA TTA ACC CCG TTG GCT TAC AAG	144
Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys	
35 40 45	
25 CAA TTC ATC CCC AAC GTT GCT GAG AAA ACG CTT GGA GCC AGC GGC AAA	192
Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys	
50 55 60	
30 TAC GAA GGC AAA ATC ACA AGG AAT TCA GAG AGA TTT AAA GAG CTG ATT	240
Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile	
65 70 75 80	
35 CCG AAT TAT AAT CCC GAT ATC ATC TTT AAG GAC GAG GAA AAC ACA AAC	288
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn	
85 90 95	
40 GCT GAC AGG CTG ATG ACC AAG CGC TGT AAG GAC AAG TTA AAT TCG TTG	336
Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu	
100 105 110	
45 GCC ATA TCC GTC ATG AAC CAC TGG CCC GGC GTG AAA CTG CGC GTC ACT	384
Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr	
115 120 125	
50 GAA GGC TGG GAT GAG GAT GGT CAC CAT TTA GAA GAA TCT TTG CAC TAT	432
Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr	
130 135 140	
55 GAG GGA CGG GCA GTG GAC ATC ACT ACC TCA GAC AGG GAT AAA AGC AAG	480
Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys	
145 150 155 160	
60 TAT GGG ATG CTA TCC AGG CTT GCA GTG GAG GCA GGA TTC GAC TGG GTC	528
Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val	
165 170 175	
55 TAT TAT GAA TCT AAA GCC CAC ATA CAC TGC TCT GTC AAA GCA GAA AAT	576
Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn	
180 185 190	
60 TCA GTG GCT GCT AAA TCA GGA GGA TGT TTT CCT GGG TCT GGG ACG GTG	624
Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val	

	195	200	205	
5	ACA CTT GGT GAT GGG ACG AGG AAA CCC ATC AAA GAT CTT AAA GTG GGC Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly 210 215 220			672
10	GAC CGG GTT TTG GCT GCA GAC GAG AAG GGA AAT GTC TTA ATA AGC GAC Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp 225 230 235 240			720
15	TTT ATT ATG TTT ATA GAC CAC GAT CCG ACA ACG AGA AGG CAA TTC ATC Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile 245 250 255			768
20	GTC ATC GAG ACG TCA GAA CCT TTC ACC AAG CTC ACC CTC ACT GCC GCG Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala 260 265 270			816
25	CAC CTA GTT TTC GTT GGA AAC TCT TCA GCA GCT TCG GGT ATA ACA GCA His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala 275 280 285			864
30	ACA TTT GCC AGC AAC GTG AAG CCT GGA GAT ACA GTT TTA GTG TGG GAA Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu 290 295 300			912
35	GAC ACA TGC GAG AGC CTC AAG AGC GTT ACA GTG AAA AGG ATT TAC ACT Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr 305 310 315 320			960
40	GAG GAG CAC GAG GGC TCT TTT GCG CCA GTC ACC GCG CAC GGA ACC ATA Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile 325 330 335			1008
45	ATA GTG GAT CAG GTG TTG GCA TCG TGC TAC GCG GTC ATT GAG AAC CAC Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His 340 345 350			1056
50	AAA TGG GCA CAT TGG GCT TTT GCG CCG GTC AGG TTG TGT CAC AAG CTG Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu 355 360 365			1104
55	ATG ACG TGG CTT TTT CCG GCT CGT GAA TCA AAC GTC AAT TTT CAG GAG Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu 370 375 380			1152
60	GAT GGT ATC CAC TGG TAC TCA AAT ATG CTG TTT CAC ATC GGC TCT TGG Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp 385 390 395 400			1200
65	CTG CTG GAC AGA GAC TCT TTC CAT CCA CTC GGG ATT TTA CAC TTA AGT Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser 405 410 415			1248
70	TGA			1251

(2) INFORMATION FOR SEQ ID NO:10:

- 60 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 425 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

10	Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile	1	5	10	15
	Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly	20	25	30	
15	Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys	35	40	45	
	Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg	50	55	60	
20	Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr	65	70	75	80
	Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly	85	90	95	
25	Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu	100	105	110	
30	Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr	115	120	125	
	Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr	130	135	140	
35	Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys	145	150	155	160
	Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val	165	170	175	
40	Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn	180	185	190	
45	Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val	195	200	205	
	His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly	210	215	220	
50	Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp	225	230	235	240
	Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr	245	250	255	
55	Val Ile Glu Thr Arg Gln Pro Arg Ala Arg Leu Leu Leu Thr Ala Ala	260	265	270	
60	His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly	275	280	285	

Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln
 290 295 300

5 Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser
 305 310 315 320

Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro
 325 330 335

10 Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys
 340 345 350

Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro
 355 360 365

15 Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala
 370 375 380

20 Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg
 385 390 395 400

Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His
 405 410 415

25 Pro Leu Gly Met Val Ala Pro Ala Ser
 420 425

30 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 396 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Ala Leu Pro Ala Ser Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
 1 5 10 15

45 Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
 20 25 30

Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
 35 40 45

50 Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
 50 55 60

55 Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
 65 70 75 80

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
 85 90 95

60 Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile

	100	105	110
	Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly		
	115	120	125
5	Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly		
	130	135	140
10	Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly		
	145	150	155
	Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr		
	165	170	175
15	Glu Ser Arg Asn His Ile His Val Ser Val Lys Ala Asp Asn Ser Leu		
	180	185	190
	Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu		
	195	200	205
20	Arg Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp		
	210	215	220
25	Val Leu Ala Ala Asp Ala Ala Gly Arg Val Val Pro Thr Pro Val Leu		
	225	230	235
	Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val		
	245	250	255
30	Glu Thr Glu Arg Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu		
	260	265	270
	Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro		
	275	280	285
35	Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly		
	290	295	300
40	Gly Asp Ala Leu Gln Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu		
	305	310	315
	Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val		
	325	330	335
45	Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp		
	340	345	350
	Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala		
	355	360	365
50	Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser		
	370	375	380
55	Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly		
	385	390	395

(2) INFORMATION FOR SEQ ID NO:12:

60

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 411 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

10	Met	Ser	Pro	Ala	Trp	Leu	Arg	Pro	Arg	Leu	Arg	Phe	Cys	Leu	Phe	Leu	1	5	10	15
	Leu	Leu	Leu	Leu	Leu	Val	Pro	Ala	Ala	Arg	Gly	Cys	Gly	Pro	Gly	Arg	20	25	30	
15	Val	Val	Gly	Ser	Arg	Arg	Arg	Pro	Pro	Arg	Lys	Leu	Val	Pro	Leu	Ala	35	40	45	
20	Tyr	Lys	Gln	Phe	Ser	Pro	Asn	Val	Pro	Glu	Lys	Thr	Leu	Gly	Ala	Ser	50	55	60	
	Gly	Arg	Tyr	Glu	Gly	Lys	Ile	Ala	Arg	Ser	Ser	Glu	Arg	Phe	Lys	Glu	65	70	75	80
25	Leu	Thr	Pro	Asn	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	85	90	95	
	Thr	Gly	Ala	Asp	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Arg	Leu	Asn	100	105	110	
30	Ser	Leu	Ala	Ile	Ser	Val	Met	Asn	Gln	Trp	Pro	Gly	Val	Lys	Leu	Arg	115	120	125	
35	Val	Thr	Glu	Gly	Arg	Asp	Glu	Asp	Gly	His	His	Ser	Glu	Glu	Ser	Leu	130	135	140	
	His	Tyr	Glu	Gly	Arg	Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Arg	145	150	155	160
40	Asn	Lys	Tyr	Gly	Leu	Leu	Ala	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	165	170	175	
	Trp	Val	Tyr	Tyr	Glu	Ser	Lys	Ala	His	Val	His	Cys	Ser	Val	Lys	Ser	180	185	190	
45	Glu	His	Ser	Ala	Ala	Ala	Lys	Thr	Gly	Gly	Cys	Phe	Pro	Ala	Gly	Ala	195	200	205	
50	Gln	Val	Arg	Leu	Glu	Asn	Gly	Glu	Arg	Val	Ala	Leu	Ser	Ala	Val	Lys	210	215	220	
	Pro	Gly	Asp	Arg	Val	Leu	Ala	Met	Gly	Glu	Asp	Gly	Thr	Pro	Thr	Phe	225	230	235	240
55	Ser	Asp	Val	Leu	Ile	Phe	Leu	Asp	Arg	Glu	Pro	Asn	Arg	Leu	Arg	Ala	245	250	255	
	Phe	Gln	Val	Ile	Glu	Thr	Gln	Asp	Pro	Pro	Arg	Arg	Leu	Ala	Leu	Thr	260	265	270	
60	Pro	Ala	His	Leu	Leu	Phe	Ile	Ala	Asp	Asn	His	Thr	Glu	Pro	Ala	Ala				

		275			280			285											
		His	Phe	Arg	Ala	Thr	Phe	Ala	Ser	His	Val	Gln	Pro	Gly	Gln	Tyr	Val		
		290						295					300						
5		Leu	Val	Ser	Gly	Val	Pro	Gly	Leu	Gln	Pro	Ala	Arg	Val	Ala	Ala	Val		
		305					310					315					320		
		Ser	Thr	His	Val	Ala	Leu	Gly	Ser	Tyr	Ala	Pro	Leu	Thr	Arg	His	Gly		
10					325						330					335			
		Thr	Leu	Val	Val	Glu	Asp	Val	Val	Ala	Ser	Cys	Phe	Ala	Ala	Val	Ala		
					340					345					350				
15		Asp	His	His	Leu	Ala	Gln	Leu	Ala	Phe	Trp	Pro	Leu	Arg	Leu	Phe	Pro		
				355					360					365					
		Ser	Leu	Ala	Trp	Gly	Ser	Trp	Thr	Pro	Ser	Glu	Gly	Val	His	Ser	Tyr		
		370						375					380						
20		Pro	Gln	Met	Leu	Tyr	Arg	Leu	Gly	Arg	Leu	Leu	Leu	Glu	Glu	Ser	Thr		
		385					390					395					400		
		Phe	His	Pro	Leu	Gly	Met	Ser	Gly	Ala	Gly	Ser							
25					405						410								

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 437 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

	Met	Leu	Leu	Leu	Leu	Ala	Arg	Cys	Phe	Leu	Val	Ile	Leu	Ala	Ser	Ser
	1				5					10					15	
45	Leu	Leu	Val	Cys	Pro	Gly	Leu	Ala	Cys	Gly	Pro	Gly	Arg	Gly	Phe	Gly
				20					25					30		
	Lys	Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe
				35				40					45			
50	Ile	Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu
		50					55					60				
	Gly	Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn
55	65					70					75					80
	Tyr	Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp
					85					90					95	
60	Arg	Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ala	Leu	Ala	Ile
				100					105					110		

Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
 115 120 125
 5 Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly
 130 135 140
 Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly
 145 150 155 160
 10 Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
 165 170 175
 Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val
 180 185 190
 15 Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu
 195 200 205
 20 Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg
 210 215 220
 Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu
 225 230 235 240
 25 Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile
 245 250 255
 30 Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu
 260 265 270
 Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser
 275 280 285
 35 Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val
 290 295 300
 Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser
 305 310 315 320
 40 Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala
 325 330 335
 His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val
 340 345 350
 45 Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu
 355 360 365
 50 Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly
 370 375 380
 Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly
 385 390 395 400
 55 Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His
 405 410 415
 60 Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met
 420 425 430

Ala Val Lys Ser Ser
435

5 (2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 418 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met	Arg	Leu	Leu	Thr	Arg	Val	Leu	Leu	Val	Ser	Leu	Leu	Thr	Leu	Ser	1	5	10	15
Leu	Val	Val	Ser	Gly	Leu	Ala	Cys	Gly	Pro	Gly	Arg	Gly	Tyr	Gly	Arg	20	25	30	
Arg	Arg	His	Pro	Lys	Lys	Leu	Thr	Pro	Leu	Ala	Tyr	Lys	Gln	Phe	Ile	25	35	40	45
Pro	Asn	Val	Ala	Glu	Lys	Thr	Leu	Gly	Ala	Ser	Gly	Arg	Tyr	Glu	Gly	50	55	60	
Lys	Ile	Thr	Arg	Asn	Ser	Glu	Arg	Phe	Lys	Glu	Leu	Thr	Pro	Asn	Tyr	30	65	70	75
Asn	Pro	Asp	Ile	Ile	Phe	Lys	Asp	Glu	Glu	Asn	Thr	Gly	Ala	Asp	Arg	85	90	95	
Leu	Met	Thr	Gln	Arg	Cys	Lys	Asp	Lys	Leu	Asn	Ser	Leu	Ala	Ile	Ser	35	100	105	110
Val	Met	Asn	His	Trp	Pro	Gly	Val	Lys	Leu	Arg	Val	Thr	Glu	Gly	Trp	40	115	120	125
Asp	Glu	Asp	Gly	His	His	Phe	Glu	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	130	135	140	
Ala	Val	Asp	Ile	Thr	Thr	Ser	Asp	Arg	Asp	Lys	Ser	Lys	Tyr	Gly	Thr	45	145	150	155
Leu	Ser	Arg	Leu	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	160	165	170	175
Ser	Lys	Ala	His	Ile	His	Cys	Ser	Val	Lys	Ala	Glu	Asn	Ser	Val	Ala	50	180	185	190
Ala	Lys	Ser	Gly	Gly	Cys	Phe	Pro	Gly	Ser	Ala	Leu	Val	Ser	Leu	Gln	55	195	200	205
Asp	Gly	Gly	Gln	Lys	Ala	Val	Lys	Asp	Leu	Asn	Pro	Gly	Asp	Lys	Val	210	215	220	
Leu	Ala	Ala	Asp	Ser	Ala	Gly	Asn	Leu	Val	Phe	Ser	Asp	Phe	Ile	Met	60	225	230	235

[illegible]

40 (2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 475 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
1 5 10 15

55 Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
 20 25 30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
35 40 45

60 Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly

	50		55		60
	Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr				
	65		70		75 80
5	Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg				
		85		90	95
10	Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser				
		100		105	110
	Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp				
		115		120	125
15	Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg				
		130		135	140
	Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met				
		145		150	155 160
20	Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu				
		165		170	175
	Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala				
25		180		185	190
	Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu				
		195		200	205
30	Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val				
		210		215	220
	Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr				
		225		230	235 240
35	Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu				
		245		250	255
	Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu				
40		260		265	270
	Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser				
		275		280	285
45	Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu				
		290		295	300
	Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu				
		305		310	315 320
50	Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr				
		325		330	335
	Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly				
55		340		345	350
	Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu				
		355		360	365
60	Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His				
		370		375	380

Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp
385 390 395 400

5 Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr
405 410 415

Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile
420 425 430

10 His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp
435 440 445

Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser
15 450 455 460

Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala
465 470 475

20 (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: amino acid
25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

30 Met Ser Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu
1 5 10 15

Leu Leu Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg
35 20 25 30

Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
35 40 45

40 Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
50 55 60

Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
45 65 70 75 80

Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
85 90 95

Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
50 100 105 110

Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
115 120 125

55 Val Thr Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
130 135 140

His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
145 150 155 160

60 Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp

	165	170	175
	Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser		
	180	185	190
5	Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala		
	195	200	205
	Gln Val Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg		
10	210	215	220
	Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe		
	225	230	235
15	Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala		
	245	250	255
	Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr		
20	260	265	270
	Pro Ala His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala		
	275	280	285
	Arg Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val		
25	290	295	300
	Leu Val Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val		
	305	310	315
30	Ser Thr His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly		
	325	330	335
	Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala		
35	340	345	350
	Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His		
	355	360	365
	Ser Leu Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr		
40	370	375	380
	Pro Gln Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser		
	385	390	395
45	Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser		
	405	410	

(2). INFORMATION FOR SEQ ID NO:17:

50	(i) SEQUENCE CHARACTERISTICS:
	(A) LENGTH: 396 amino acids
	(B) TYPE: amino acid
	(D) TOPOLOGY: linear
55	(ii) MOLECULE TYPE: protein
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
60	Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
	1 5 10 15

Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
 20 25 30
 5 Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
 35 40 45
 Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
 50 55 60
 10 Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
 65 70 75 80
 Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
 85 90 95
 15 Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
 100 105 110
 Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
 115 120 125
 Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
 130 135 140
 25 Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
 145 150 155 160
 Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
 165 170 175
 30 Glu Ser Arg Asn His Val His Val Ser Val Lys Ala Asp Asn Ser Leu
 180 185 190
 Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
 195 200 205
 Trp Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
 210 215 220
 40 Val Leu Ala Ala Asp Ala Ser Gly Arg Val Val Pro Thr Pro Val Leu
 225 230 235 240
 Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
 245 250 255
 45 Glu Thr Glu Trp Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
 260 265 270
 Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
 275 280 285
 Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
 290 295 300
 55 Gly Asp Ala Leu Arg Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
 305 310 315 320
 Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
 325 330 335
 60 Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp

340 345 350

Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
 355 360 365

5 Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
 370 375 380

10 Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Leu Gly
 385 390 395

(2) INFORMATION FOR SEQ ID NO:18:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 416 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

25 Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
 1 5 10 15

Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly
 20 25 30

30 Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
 35 40 45

Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys
 35 50 55 60

Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile
 65 70 75 80

40 Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn
 85 90 95

Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu
 100 105 110

45 Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr
 115 120 125

Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr
 50 130 135 140

Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys
 145 150 155 160

55 Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
 165 170 175

Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
 180 185 190

60 Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val

	195		200		205
	Thr Leu Gly Asp Gly	Thr Arg Lys Pro Ile Lys Asp	Leu Lys Val Gly		
	210	215	220		
5	Asp Arg Val Leu Ala	Ala Asp Glu Lys Gly	Asn Val Leu Ile Ser Asp		
	225	230	235	240	
	Phe Ile Met Phe Ile	Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile			
10		245	250	255	
	Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala				
	260	265	270		
15	His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala				
	275	280	285		
	Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu				
20	290	295	300		
	Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr				
	305	310	315	320	
	Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile				
25		325	330	335	
	Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His				
	340	345	350		
30	Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu				
	355	360	365		
	Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu				
35	370	375	380		
	Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp				
	385	390	395	400	
	Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser				
40		405	410	415	

(2) INFORMATION FOR SEQ ID NO:19:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1416 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

55 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 1..1413

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATG GAT AAC CAC AGC TCA GTG CCT TGG GCC AGT GCC GCC AGT GTC ACC

48

	Met	Asp	Asn	His	Ser	Ser	Val	Pro	Trp	Ala	Ser	Ala	Ala	Ser	Val	Thr	
	1				5					10					15		
5	TGT	CTC	TCC	CTG	GGA	TGC	CAA	ATG	CCA	CAG	TTC	CAG	TTC	CAG	TTC	CAG	96
	Cys	Leu	Ser	Leu	Gly	Cys	Gln	Met	Pro	Gln	Phe	Gln	Phe	Gln	Phe	Gln	
				20					25					30			
10	CTC	CAA	ATC	CGC	AGC	GAG	CTC	CAT	CTC	CGC	AAG	CCC	GCA	AGA	AGA	ACG	144
	Leu	Gln	Ile	Arg	Ser	Glu	Leu	His	Leu	Arg	Lys	Pro	Ala	Arg	Arg	Thr	
				35				40					45				
15	CAA	ACG	ATG	CGC	CAC	ATT	GCG	CAT	ACG	CAG	CGT	TGC	CTC	AGC	AGG	CTG	192
	Gln	Thr	Met	Arg	His	Ile	Ala	His	Thr	Gln	Arg	Cys	Leu	Ser	Arg	Leu	
			50				55					60					
20	ACC	TCT	CTG	GTG	GCC	CTG	CTG	CTG	ATC	GTC	TTG	CCG	ATG	GTC	TTT	AGC	240
	Thr	Ser	Leu	Val	Ala	Leu	Leu	Leu	Ile	Val	Leu	Pro	Met	Val	Phe	Ser	
	65					70					75					80	
25	CCG	GCT	CAC	AGC	TGC	GGT	CCT	GGC	CGA	GGA	TTG	GGT	CGT	CAT	AGG	GCG	288
	Pro	Ala	His	Ser	Cys	Gly	Pro	Gly	Arg	Gly	Leu	Gly	Arg	His	Arg	Ala	
					85				90						95		
30	CGC	AAC	CTG	TAT	CCG	CTG	GTC	CTC	AAG	CAG	ACA	ATT	CCC	AAT	CTA	TCC	336
	Arg	Asn	Leu	Tyr	Pro	Leu	Val	Leu	Lys	Gln	Thr	Ile	Pro	Asn	Leu	Ser	
				100					105					110			
35	GAG	TAC	ACG	AAC	AGC	GCC	TCC	GGA	CCT	CTG	GAG	GGT	GTG	ATC	CGT	CGG	384
	Glu	Tyr	Thr	Asn	Ser	Ala	Ser	Gly	Pro	Leu	Glu	Gly	Val	Ile	Arg	Arg	
				115				120					125				
40	GAT	TCG	CCC	AAA	TTC	AAG	GAC	CTC	GTG	CCC	AAC	TAC	AAC	AGG	GAC	ATC	432
	Asp	Ser	Pro	Lys	Phe	Lys	Asp	Leu	Val	Pro	Asn	Tyr	Asn	Arg	Asp	Ile	
			130				135					140					
45	CTT	TTC	CGT	GAC	GAG	GAA	GGC	ACC	GGA	GCG	GAT	GGC	TTG	ATG	AGC	AAG	480
	Leu	Phe	Arg	Asp	Glu	Glu	Gly	Thr	Gly	Ala	Asp	Gly	Leu	Met	Ser	Lys	
	145					150					155					160	
50	CGC	TGC	AAG	GAG	AAG	CTA	AAC	GTG	CTG	GCC	TAC	TCG	GTG	ATG	AAC	GAA	528
	Arg	Cys	Lys	Glu	Lys	Leu	Asn	Val	Leu	Ala	Tyr	Ser	Val	Met	Asn	Glu	
					165				170						175		
55	TGG	CCC	GGC	ATC	CGG	CTG	CTG	GTC	ACC	GAG	AGC	TGG	GAC	GAG	GAC	TAC	576
	Trp	Pro	Gly	Ile	Arg	Leu	Leu	Val	Thr	Glu	Ser	Trp	Asp	Glu	Asp	Tyr	
				180					185					190			
60	CAT	CAC	GGC	CAG	GAG	TCG	CTC	CAC	TAC	GAG	GGC	CGA	GCG	GTG	ACC	ATT	624
	His	His	Gly	Gln	Glu	Ser	Leu	His	Tyr	Glu	Gly	Arg	Ala	Val	Thr	Ile	
				195			200						205				
65	GCC	ACC	TCC	GAT	CGC	GAC	CAG	TCC	AAA	TAC	GGC	ATG	CTC	GCT	CGC	CTG	672
	Ala	Thr	Ser	Asp	Arg	Asp	Gln	Ser	Lys	Tyr	Gly	Met	Leu	Ala	Arg	Leu	
				210			215					220					
70	GCC	GTC	GAG	GCT	GGA	TTC	GAT	TGG	GTC	TCC	TAC	GTC	AGC	AGG	CGC	CAC	720
	Ala	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Ser	Tyr	Val	Ser	Arg	Arg	His	
	225					230					235					240	
75	ATC	TAC	TGC	TCC	GTC	AAG	TCA	GAT	TCG	TCG	ATC	AGT	TCC	CAC	GTG	CAC	768
	Ile	Tyr	Cys	Ser	Val	Lys	Ser	Asp	Ser	Ser	Ile	Ser	Ser	Ser	His	Val	

	245	250	255	
5	GGC TGC TTC ACG CCG GAG AGC ACA GCG CTG CTG GAG AGT GGA GTC CGG Gly Cys Phe Thr Pro Glu Ser Thr Ala Leu Leu Glu Ser Gly Val Arg 260 265 270	816		
10	AAG CCG CTC GGC GAG CTC TCT ATC GGA GAT CGT GTT TTG AGC ATG ACC Lys Pro Leu Gly Glu Leu Ser Ile Gly Asp Arg Val Leu Ser Met Thr 275 280 285	864		
15	GCC AAC GGA CAG GCC GTC TAC AGC GAA GTG ATC CTC TTC ATG GAC CGC Ala Asn Gly Gln Ala Val Tyr Ser Glu Val Ile Leu Phe Met Asp Arg 290 295 300	912		
20	AAC CTC GAG CAG ATG CAA AAC TTT GTG CAG CTG CAC ACG GAC GGT GGA Asn Leu Glu Gln Met Gln Asn Phe Val Gln Leu His Thr Asp Gly Gly 305 310 315 320	960		
25	GCA GTG CTC ACG GTG ACG CCG GCT CAC CTG GTT AGC GTT TGG CAG CCG Ala Val Leu Thr Val Thr Pro Ala His Leu Val Ser Val Trp Gln Pro 325 330 335	1008		
30	GAG AGC CAG AAG CTC ACG TTT GTG TTT GCG CAT CGC ATC GAG GAG AAG Glu Ser Gln Lys Leu Thr Phe Val Phe Ala His Arg Ile Glu Glu Lys 340 345 350	1056		
35	AAC CAG GTG CTC GTA CCG GAT GTG GAG ACG GGC GAG CTG AGG CCC CAG Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln 355 360 365	1104		
40	CGA GTG GTC AAG TTG GGC AGT GTG CGC AGT AAG GGC GTG GTC GCG CCG Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro 370 375 380	1152		
45	CTG ACC CGC GAG GGC ACC ATT GTG GTC AAC TCG GTG GCC GCC AGT TGC Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys 385 390 395 400	1200		
50	TAT GCG GTG ATC AAC AGT CAG TCG CTG GCC CAC TGG GGA CTG GCT CCC Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro 405 410 415	1248		
55	ATG CGC CTG CTG TCC ACG CTG GAG GCG TGG CTG CCC GCC AAG GAG CAG Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln 420 425 430	1296		
60	TTG CAC AGT TCG CCG AAG GTG GTG AGC TCG GCG CAG CAG CAG AAT GGC Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly 435 440 445	1344		
65	ATC CAT TGG TAT GCC AAT GCG CTC TAC AAG GTC AAG GAC TAC GTG CTG Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu 450 455 460	1392		
70	CCG CAG AGC TGG CGC CAC GAT TGA Pro Gln Ser Trp Arg His Asp 465 470	1416		
75	(2) INFORMATION FOR SEQ ID NO:20:			

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 471 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

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10 Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
    1          5          10          15
    Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln
        20          25          30
15 Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr
    35          40          45
    Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu
    50          55          60
    Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser
    65          70          75          80
25 Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala
    85          90          95
    Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser
    100          105          110
30 Glu Tyr Thr Asn Ser Ala Ser Gly Pro Leu Glu Gly Val Ile Arg Arg
    115          120          125
    Asp Ser Pro Lys Phe Lys Asp Leu Val Pro Asn Tyr Asn Arg Asp Ile
    130          135          140
    Leu Phe Arg Asp Glu Glu Gly Thr Gly Ala Asp Gly Leu Met Ser Lys
    145          150          155          160
40 Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn Glu
    165          170          175
    Trp Pro Gly Ile Arg Leu Leu Val Thr Glu Ser Trp Asp Glu Asp Tyr
    180          185          190
45 His His Gly Gln Glu Ser Leu His Tyr Glu Gly Arg Ala Val Thr Ile
    195          200          205
    Ala Thr Ser Asp Arg Asp Gln Ser Lys Tyr Gly Met Leu Ala Arg Leu
    210          215          220
    Ala Val Glu Ala Gly Phe Asp Trp Val Ser Tyr Val Ser Arg Arg His
    225          230          235          240
55 Ile Tyr Cys Ser Val Lys Ser Asp Ser Ser Ile Ser Ser His Val His
    245          250          255
    Gly Cys Phe Thr Pro Glu Ser Thr Ala Leu Leu Glu Ser Gly Val Arg
    260          265          270
60 Lys Pro Leu Gly Glu Leu Ser Ile Gly Asp Arg Val Leu Ser Met Thr

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275 280 285
 Ala Asn Gly Gln Ala Val Tyr Ser Glu Val Ile Leu Phe Met Asp Arg
 290 295 300
 5 Asn Leu Glu Gln Met Gln Asn Phe Val Gln Leu His Thr Asp Gly Gly
 305 310 315 320
 10 Ala Val Leu Thr Val Thr Pro Ala His Leu Val Ser Val Trp Gln Pro
 325 330 335
 Glu Ser Gln Lys Leu Thr Phe Val Phe Ala His Arg Ile Glu Glu Lys
 340 345 350
 15 Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln
 355 360 365
 Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro
 370 375 380
 20 Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys
 385 390 395 400
 Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro
 405 410 415
 25 Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln
 420 425 430
 30 Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly
 435 440 445
 Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu
 450 455 460
 35 Pro Gln Ser Trp Arg His Asp
 465 470
 40 (2) INFORMATION FOR SEQ ID NO:21:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 221 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 45 (ii) MOLECULE TYPE: peptide
 (v) FRAGMENT TYPE: internal
 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
 55 Cys Gly Pro Gly Arg Gly Xaa Gly Xaa Arg Arg His Pro Lys Lys Leu
 1 5 10 15
 Thr Pro Leu Ala Tyr Lys Gln Phe Ile Pro Asn Val Ala Glu Lys Thr
 20 25 30
 60 Leu Gly Ala Ser Gly Arg Tyr Glu Gly Lys Ile Xaa Arg Asn Ser Glu
 35 40 45

Arg Phe Lys Glu Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys
 50 55 60
 5 Asp Glu Glu Asn Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys
 65 70 75 80
 Asp Lys Leu Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp Pro Gly
 85 90 95
 10 Val Xaa Leu Arg Val Thr Glu Gly Trp Asp Glu Asp Gly His His Xaa
 100 105 110
 Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser
 115 120 125
 15 Asp Arg Asp Xaa Ser Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala Val Glu
 130 135 140
 20 Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Lys Ala His Ile His Cys
 145 150 155 160
 Ser Val Lys Ala Glu Asn Ser Val Ala Ala Lys Ser Gly Gly Cys Phe
 165 170 175
 25 Pro Gly Ser Ala Xaa Val Xaa Leu Xaa Xaa Gly Gly Xaa Lys Xaa Val
 180 185 190
 Lys Asp Leu Xaa Pro Gly Asp Xaa Val Leu Ala Ala Asp Xaa Xaa Gly
 195 200 205
 30 Xaa Leu Xaa Xaa Ser Asp Phe Xaa Xaa Phe Xaa Asp Arg
 210 215 220

35

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 167 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: peptide

45

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

50

Cys Gly Pro Gly Arg Gly Xaa Xaa Xaa Arg Arg Xaa Xaa Xaa Pro Lys
 1 5 10 15

55

Xaa Leu Xaa Pro Leu Xaa Tyr Lys Gln Phe Xaa Pro Xaa Xaa Xaa Glu
 20 25 30

Xaa Thr Leu Gly Ala Ser Gly Xaa Xaa Glu Gly Xaa Xaa Xaa Arg Xaa
 35 40 45

60

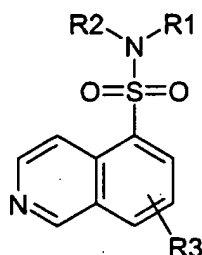
Ser Glu Arg Phe Xaa Xaa Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile
 50 55 60

	Phe	Lys	Asp	Glu	Glu	Asn	Xaa	Gly	Ala	Asp	Arg	Leu	Met	Thr	Xaa	Arg	
	65					70					75					80	
5	Cys	Lys	Xaa	Xaa	Xaa	Asn	Xaa	Leu	Ala	Ile	Ser	Val	Met	Asn	Xaa	Trp	
					85					90					95		
	Pro	Gly	Val	Xaa	Leu	Arg	Val	Thr	Glu	Gly	Xaa	Asp	Glu	Asp	Gly	His	
				100					105						110		
10	His	Xaa	Xaa	Xaa	Ser	Leu	His	Tyr	Glu	Gly	Arg	Ala	Xaa	Asp	Ile	Thr	
			115					120						125			
	Thr	Ser	Asp	Arg	Asp	Xaa	Xaa	Lys	Tyr	Gly	Xaa	Leu	Xaa	Arg	Leu	Ala	
15			130					135						140			
	Val	Glu	Ala	Gly	Phe	Asp	Trp	Val	Tyr	Tyr	Glu	Ser	Xaa	Xaa	His	Xaa	
	145					150					155					160	
20	His	Xaa	Ser	Val	Lys	Xaa	Xaa										
					165												

We claim:

1. A method for modulating the immune function of an animal comprising administering to the animal a therapeutic amount of a *hedgehog* or *ptc* therapeutic.
- 5 2. A method for suppressing the immune system of an animal comprising contacting the cells with an effective amount of a *hedgehog* protein, or agonist thereof.
3. A method for enhancing the immune system of an animal comprising administering to a immunostimulatory amount of a *hedgehog* antagonist.
- 10 4. The method of any of claims 1, wherein the *hedgehog* therapeutic is a polypeptide which includes a hedgehog amino acid sequence which is identical or homologous to an amino acid sequence of any one of SEQ ID Nos. 10-18.
5. The method of claim 4, wherein the hedgehog amino acid sequence is sufficient for specific binding of the polypeptide to a *patched* protein.
- 15 6. The method of claim 4, wherein the hedgehog amino acid sequence is at least 80 percent identical to an amino acid sequence of any one of SEQ ID Nos. 10-18.
7. The method of claim 4, wherein the hedgehog amino acid sequence is encodable by a nucleic acid which hybridizes under stringent conditions to any one of SEQ ID Nos. 1-9.
- 20 8. The method of claim 4, wherein the hedgehog amino acid sequence is of a vertebrate hedgehog protein.
9. The method of claim 4, wherein the polypeptide includes at least a 50 amino acid extracellular portion of a vertebrate hedgehog protein.
10. The method of claim 4, wherein the polypeptide includes at least an extracellular portion of a vertebrate hedgehog protein corresponding to residues 24-194 of SEQ ID
25 No:15.
11. The method of claim 4, wherein the hedgehog polypeptide is modified with one or more lipophilic moieties.
12. The method of claim 11, wherein the hedgehog polypeptide is modified with one or more sterol moieties.
- 30 13. The method of claim 12, wherein the sterol moiety is cholesterol.
14. The method of claim 11, wherein the hedgehog polypeptide is modified with one or more fatty acid moieties.
15. The method of claim 14, wherein each fatty acid moiety is independently selected from the group consisting of myristoyl, palmitoyl, stearoyl, and arachidoyl.

16. The method of claim 11, wherein the hedgehog polypeptide is modified with one or more aromatic hydrocarbons.
17. The method of claim 16, wherein each aromatic hydrocarbon is independently selected from the group consisting of benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.
18. The method of claim 11, wherein the hedgehog polypeptide is modified one or more times with a C7 - C30 alkyl or cycloalkyl.
19. The method of claim 1, wherein the *ptc* therapeutic is a small organic molecule.
20. The method of claim 19, wherein the binding of the *ptc* therapeutic to *patched* results in up- or down-regulation of *patched* and/or *gli* expression.
21. The method of claim 1, wherein the *ptc* therapeutic binds to *patched* and mimics *hedgehog*-mediated *patched* signal transduction.
22. The method of claim 19, wherein the *ptc* therapeutic is an inhibitor of protein kinase A.
23. The method of claim 22, wherein the PKA inhibitor is a 5-isoquinolinesulfonamide
24. The method of claim 22, wherein the PKA inhibitor is represented in the general formula:



wherein,

- 20 R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

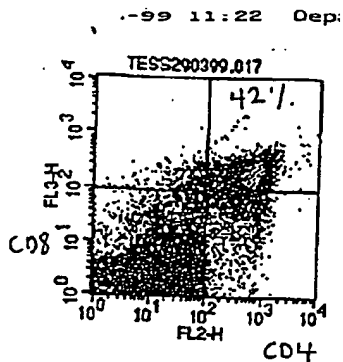
R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$ -lower alkyl, $-(CH_2)_m-O$ -lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$ -lower alkyl, $-(CH_2)_m-S$ -lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

25. The method of claim 22, wherein the PKA inhibitor is cyclic AMP analog.
26. The method of claim 22, wherein the PKA inhibitor is selected from the group consisting of N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, KT5720, 8-bromo-cAMP, dibutyryl-cAMP and PKA Heat Stable Inhibitor isoform α .
27. A therapeutic preparation of a small molecule antagonist of *patched*, which *patched* antagonist is provided in a pharmaceutically acceptable carrier and in an amount sufficient to modulate the immune system of an adult human patient.
28. A method for modulating T lymphocytes maturation, comprising administering to a patient a gene activation construct which recombines with a genomic *hedgehog* gene of the patient to provide a heterologous transcriptional regulatory sequence operatively linked to a coding sequence of the *hedgehog* gene.
29. A method of claim 2, wherein suppressing the immune function of an animal comprises inhibiting T lymphocyte maturation.
30. A method of claim 3, wherein enhancing the immune function of an animal comprises stimulating T lymphocyte maturation.



Department of Biology

E14 + 3 days culture

Neutralizing Sheet P.02

File: TESS290399.017

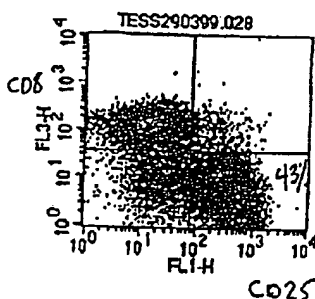
Acquisition Date: 29-Mar-99

Gate: G1

Gated Events: 15131

Quad	Events	% Gated	X Mean	Y Mean
UL	347	2.29	48.87	172.41
UR	6422	42.44	840.80	212.14
LL	6972	46.08	21.31	7.70
LR	1390	9.19	431.02	39.30

+ 47% SE1



File: TESS290399.028

Acquisition Date: 29-Mar-99

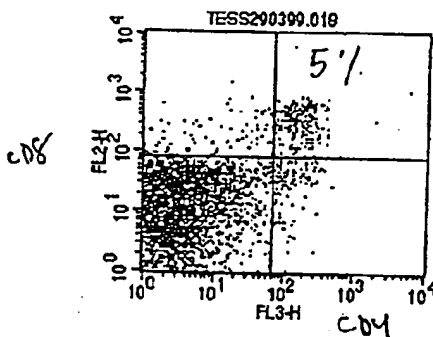
Gate: G1

Gated Events: 18580

Quad	Events	% Gated	X Mean	Y Mean
UL	7528	40.50	17.83	143.87
UR	538	2.88	288.72	148.88
LL	2443	13.14	29.20	12.27
LR	8082	43.47	421.70	9.36

+ SE1

majority these cells = SE1 have
fused with TCR rearranged



File: TESS290399.018

Acquisition Date: 29-Mar-99

Gate: G1

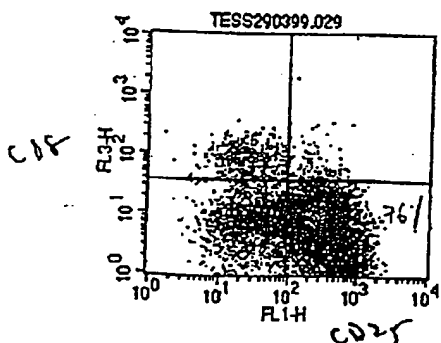
Gated Events: 4223

Quad	Events	% Gated	X Mean	Y Mean
UL	107	2.53	10.09	230.31
UR	204	4.83	201.81	389.68
LL	3803	90.05	5.08	20.84
LR	109	2.58	134.13	36.68

CD25-

medium alone
(control)

Concentrated
with TCR
rearranged
+ 14%
expression



File: TESS290399.029

Acquisition Date: 29-Mar-99

Gate: G1

Gated Events: 9775

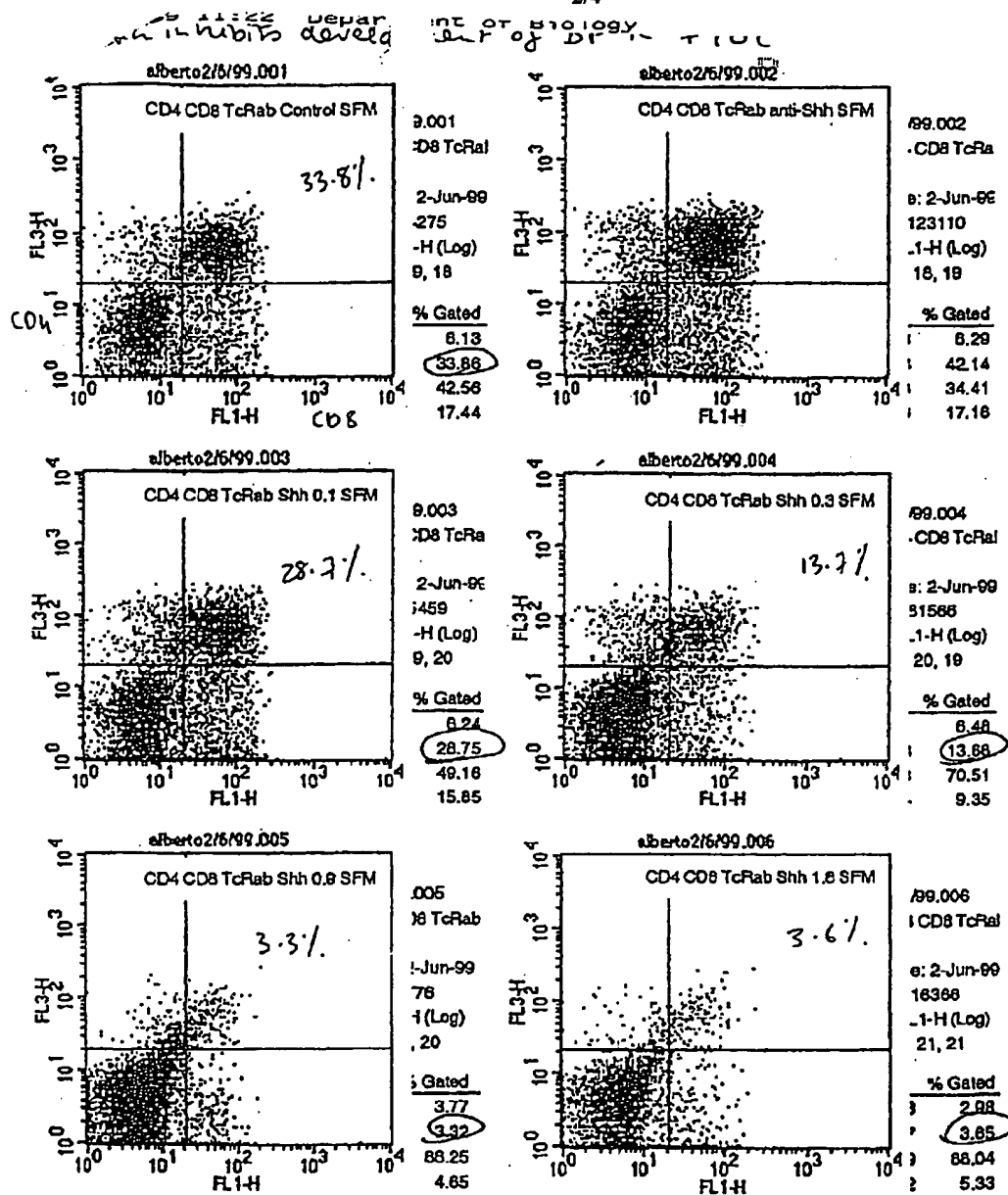
Quad	Events	% Gated	X Mean	Y Mean
UL	513	5.25	29.34	97.98
UR	142	1.45	274.89	151.41
LL	1682	17.21	38.80	9.15
LR	7438	76.09	398.43	8.81

medium alone

Effect is
dramatic &
reproducible

Accelerated development in presence of
neutralising mAb SE1.

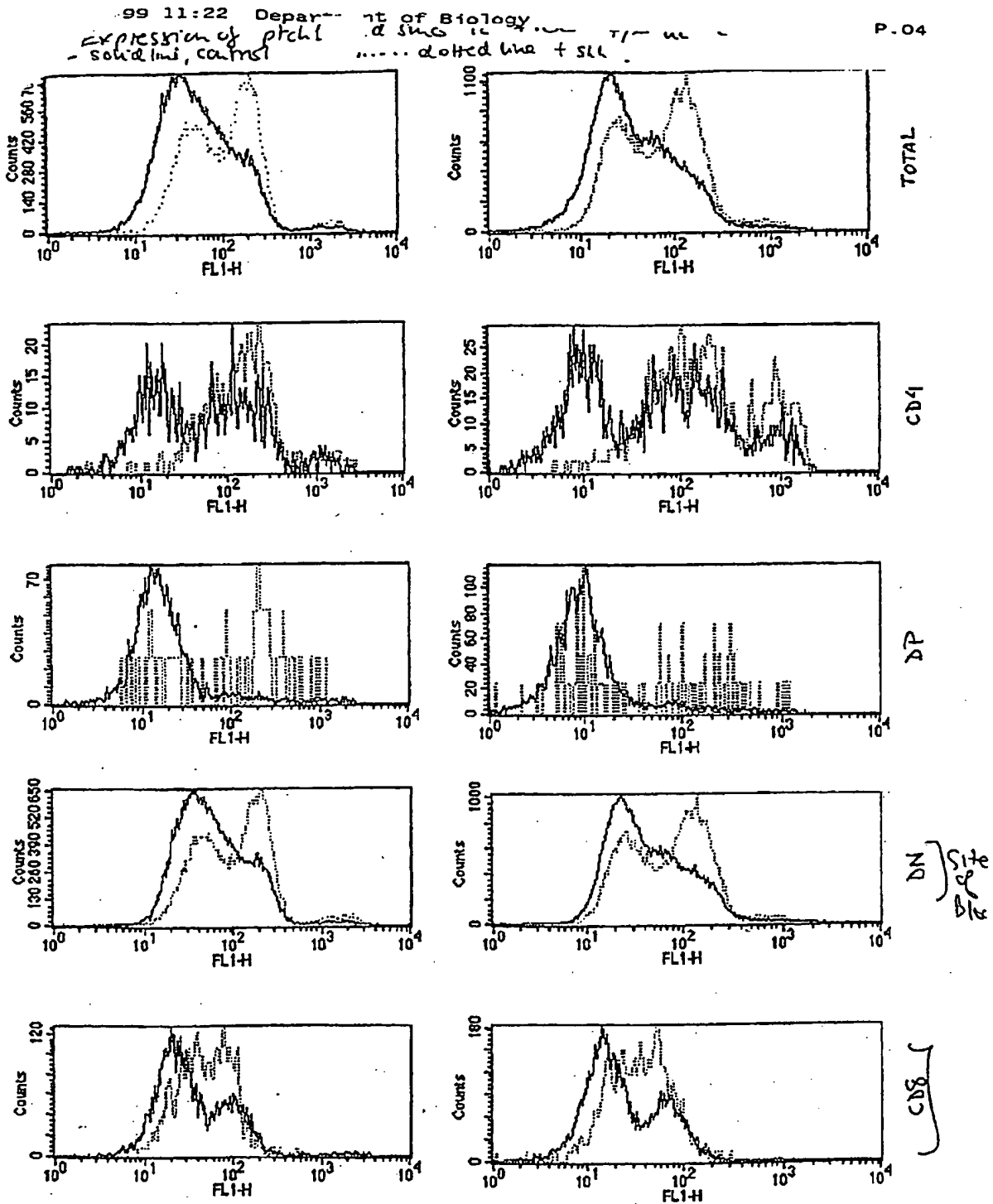
Figure 1



E14.5 + 5 days.

FTOC +5 Serum Free Medium

Figure 2



PatchG19

SmoC17

Figure 3

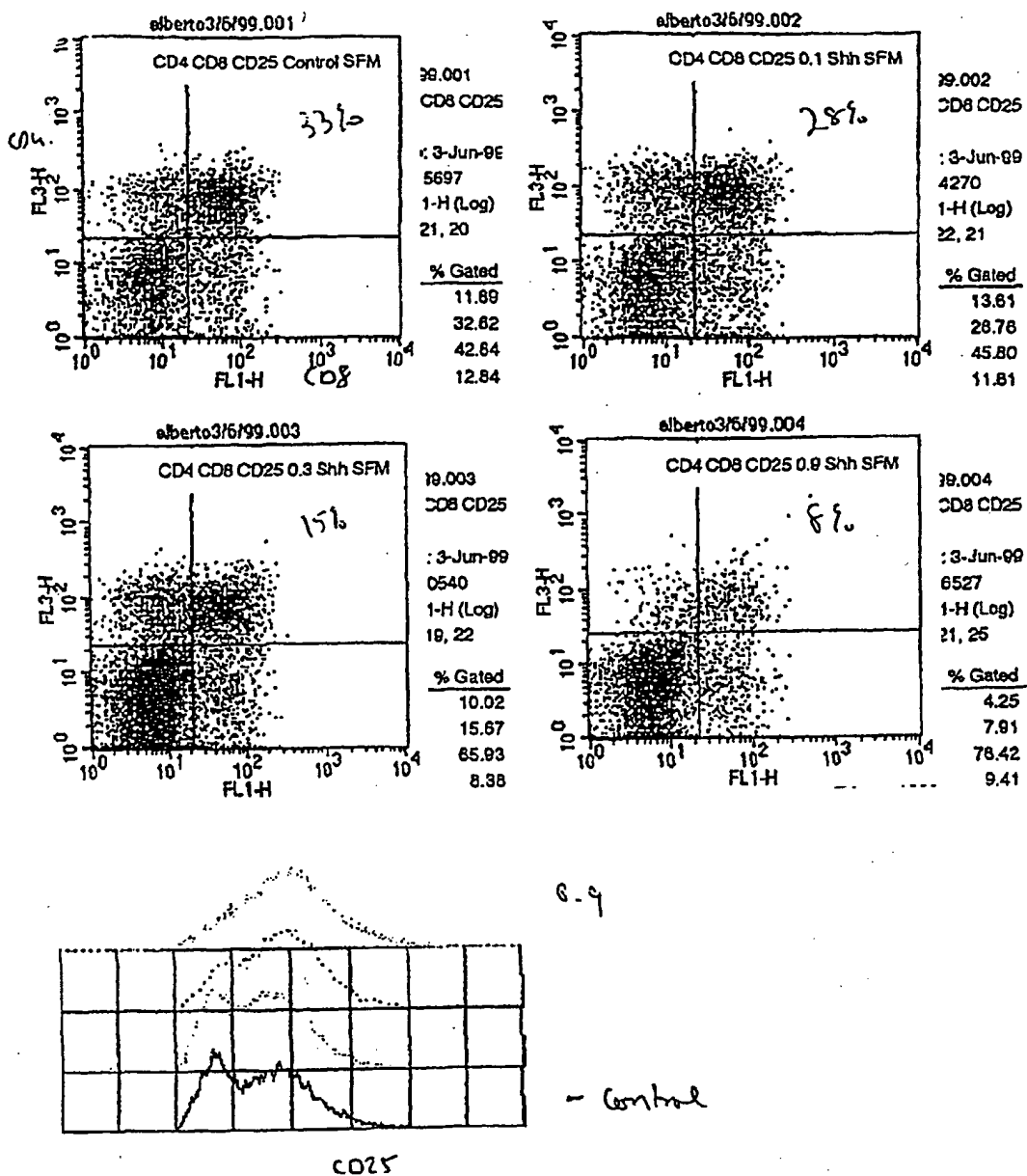


Figure 4

FTOC +6 Serum Free Medium + Shh